

Department Virology
Subject VEE
Name Parker, Michael
Address 1092 3rd St

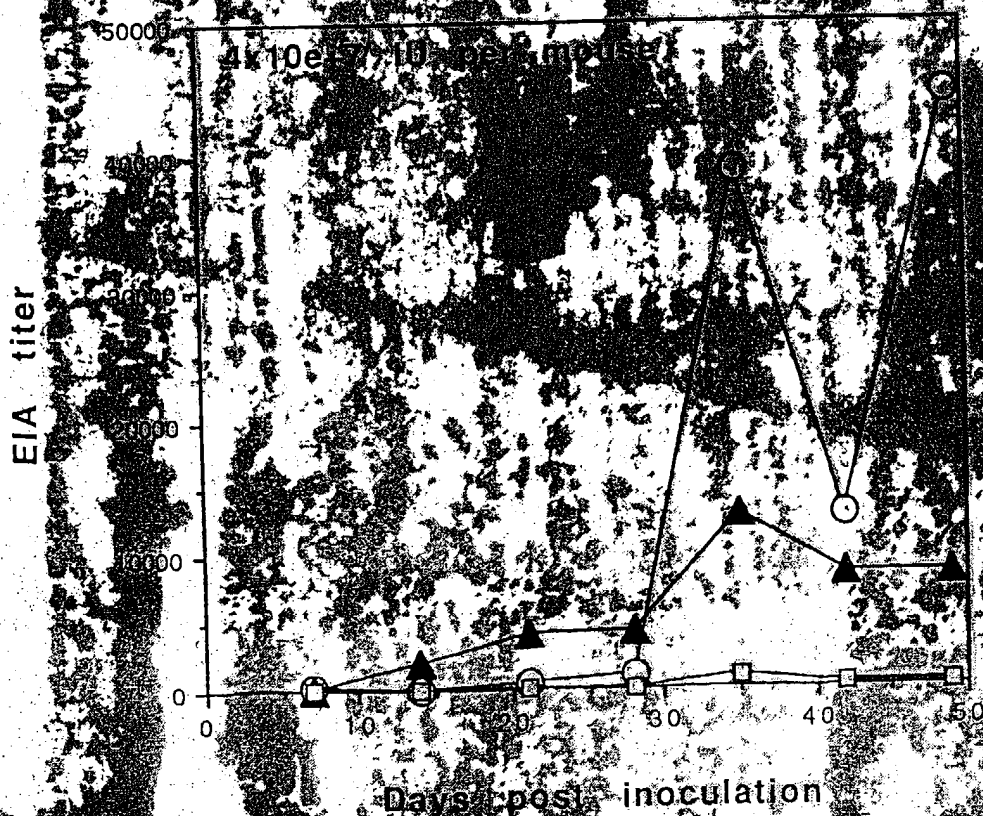
 National Brand # 3656 43-648

Computation Notebook
Dennison Stationery Products Co. Framingham, MA 01701

 Issued 75 Sheets
7-8-94 11 1/2" x 9 1/4"
4x4 Quad.

0 73333 43648 8

EXHIBIT A



7/28/1994

Transformation

BEST AVAILABLE COPY

BRL MAX Efficiency DH5 α Competent Cells

1. 10 μ l of DNA (V3000) for 50 μ l BRL DH5 α Cells
2. Keep DNA and Cells on ice (to thaw cells)
3. Put 50 μ l cells into μ tubes containing 10 μ g of V3
4. Incubate on ice for 30 min
5. Heat shock 45 seconds in 42°C, no shaking
6. Ice 2 min
7. add 1 ml of SOC (pre-heat to 37°C)
8. Shake at 37°C for 1 hour
9. Spread 100 μ l of this reaction and 900 μ l (centrifuge) on LB plates containing 100 μ g/ml Amp.

1000	①	DNA VE ^{1A strain} 272-3 (1.1 μ g/ml stock)	10 μ l containing 10 μ g (1 μ g/100 μ l)
	②	DNA	10 μ l - - 100 μ g (10 μ g/100 μ l)
	③	DNA	10 μ l - - 1 μ g (100 μ g/100 μ l)

2x 3ml overnight cultures A 3014

Clean up pV3000 Apa I + Rse II

Water	30 μ l
1.1 μ g/ml DNA	3.2 μ l
BRL REact ⁵	3.6 μ l
20 u/ml Rse II	0.5 μ l
10 u/ml Apa I	1.2 μ l

38.5 μ l $\rightarrow$ 1 hr at 37°C
 + 60 μ l of water
 + 100 μ l of phenol-chloroform
 1 min of vortex

upper phase + 10 μ l 1M NaCl + 300 μ l 95% EtOH
 -70°C o/n

cells cleaved w/ RsrII + A I isolated o/n at -70°C
spin down 15 min wash 1x no pellet
dry at the bench

resolve in
(7.5 μl) add

45 μl water

0.5 μl dNTP (50 mM)

5 μl 10x T4 Polymerase

1 μl T4 (Gibco BRL 2.9 U/μl)

~~10 μl~~

20 min

sup-
stored
in Eps. tube
and precipitating
big pellet
washed 2x
with EtOH



+ 50 μl water

+ 100 μl phenol

+ 10 μl of 1M NaCl

+ 300 μl EtOH → -70°C 20'

+ 0.5 μl tRNA

spin down, wash twice w/ 70% EtOH,
dry on the bench.

10 μl dissolve in 30 μl of water
load on the 1% agarose gel

Results of the transformation of E. coli w/ pV3000

for 50 μl of BRL Max Efficiency Comp. Cells (opt 100)

①	10 μg DNA (1 μg/μl)	10 μl total DNA vol. / 100 μl per plate	400
②	100 μg DNA (10 μg/μl)	— / —	540
③	1 μg DNA (100 μg/μl)	— / —	> 400

per plate

Results of 1% Agarose are interpreted as follows

- ① λ HindIII marker
- ② pV3000 800 ng uncut
- ③ pV3000 (ApaI & RsrII) / T4 DNA pol
- ④ pV3000 supernatant (just to check precipitation efficiency)
- ⑤ 1 kb marker

Two bands in lane ③ correspond probably
to uncut pV3000 (upper) and 6.5 kb and 7.0 kb
Concentration ~ 5 μg / 10 μl ⇒ 0.5 μg / μl

Ligation reaction

P1

DNA pV3000 as lane ③ on the gel (p. 2)
10x shake (w/ + ATP)

(2.000 µl NEB) T4 RNA ligase

15 µl

2 µl

2 µl

10°C overnight

7/30/199

Saturday

1. P1 (20 µl) + 100 µl water + 100 µl phenol,
Spin 5 min; upper phase + 10 µl 1M NaOH+ 1 µl tRNA 10.6 µg/ml
+ 400 µl 90% EtOH

20" at

Spin down
dry (speed vac)

dissolve in 7 µl water

7 µl water
1 µl RcaIT buffer
0.7 µl EcoRI 10u/ml
0.7 µl KpnI 10u/ml

37°C 15:00 - 16:00

10' at 65°C

in order to cleave plasmids resulting from
ligated linearized form (fig. on p. 2)DNA miniprep Wizard kit (from pV3000 cells)
(as in protocol of Promega) nos. 1 and 2; nos. 3. and 4. for
1% Agarose / TBE
① x wind 6 µl
② 5 µl ①
③ 5 µl ② } pV3000 purified ~200 µg/µl
④ 1 kb 6 µl ~200 µg/µl

Transformation into 100 µl of MAX efficiency comp. cells

Cleavage pV3000 ApaI + RsaII

⑤ React
DNA 45 µl (~10 µg)
10x 10 µl
ApaI 5 µl (20u/ml, neontap) + 2.5 µl (20u/ml, eunas)
RsaII 2.5 µl opus greenpura + 2.5 µl 60000
H₂O 35 µl

27°C 16:30 - 18:30

7/31/94
Sunday

P1

colonies

P1 - 100 μ l - 0 colonies

P1 - 100 μ l - 1 colony

P1 - 100 μ l - 3 colony (100)

P1 - 900 μ l - 3 colony (100)

P1: $\approx$ 7 colonies

o/n at 37 incubated 3 ml - 100 μ l

1% Agarose/TBE

① λ Hind IV

② 5 μ l of pV3000 Apa I/Rsr II

③ 1 kb

8/1/94
Monday

Kaplan's mouse *mus musculus*
K12 mouse *mus musculus*
SAS Capuscon *mus musculus*
Anaplasma *mus musculus* T4 DNA pol

① Mini-preps Nos 1-7 from P1 clones
w/ Wizard mini-prep kit 5 μ l - 1% TBE

② pV3000 cleaved w/ Apa I + Rsr II
phenol treated, + NaCl + tRNA + EoH 20' at
spin down, wash 2x 7
dry dissolve in 30 μ l water

5 μ l - 1% TBE

gel no. ②

③ Cleavage of P1 1-7 clones
mix

180 μ l H₂O

20 μ l React 2 10x

3 μ l 10 u/ μ l Xba I

3 μ l 10 u/ μ l Xba I

20 μ l each well
+ 3 μ l of DNA
37°C 1130 1400

no. ①



pV3000 Apa I/Rsr II 5 μ l

plasmids 1-4 linearized
by Xba I (Mlu ~ 2.4 kb) $\Rightarrow$
Clones are cleaved by

Cleavage Nos 1-7 and pV3000 w Sca I

mix: H₂O 180 μ l $\rightarrow$ 20 μ l
 10x React 2 20 μ l $\rightarrow$ 3 μ l of DNA
 10 μ M Sca I 15 μ l
 37°C 1 hr

T4 DNA polymerase - mediated resection

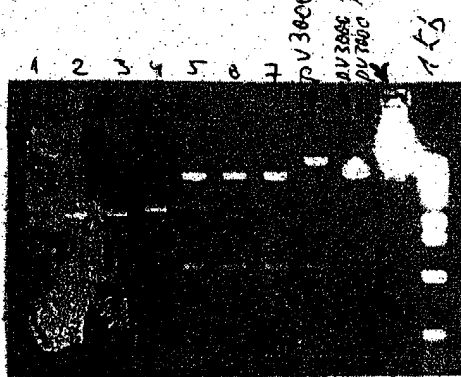
7 μ l - 1/2 Agarose/TBE

gel No. 3

pV3000 Apa I/Rsr II 45 μ l (1 μ g)
 10x T4 (P) buffer 5 μ l
 100mM dNTP 0.5 μ l
 T4 (P) 1 μ l

RT 20' + 150 μ l water
 + 200 μ l phenol - chloro
 precipitate w/ 500 μ l EtOH
 (+ NaCl + tRNA)
 20' at -70°C
 spin down wash twice &
 dissolve in 20 μ l
 5 μ l - 1/2 TBE/Agarose

no. 3



Sca I cleavage

P2

DNA 2 μ l
 H₂O 14 μ l
 10x Buf 2 μ l
 T4 2 μ l
 +10°C o/n

Nos 5, 6, 7, pV3000 o/n at 37 w/ Sph I (gel. No. 4)

8/2/9
 Tuesday

Transformation of Max Efficiency (M1) cells

- ① control pV3000/Apa I/Rsr II/T4
- ② P2 (pV3000/Apa I/Rsr II/T4/Ligase)
- ③ P2 sure (— — —)

1/2 Agarose nos 5-7 and pV3000 Sph I



gel No.

cleavage
gel ①

	5	X	X	X
	6	X	X	X
	7	X	X	X
pV 3000	X	X	X	X

no cleavage!
(or one site)

see also gel no ④ p. 5

gel no ③, p. 5

Xba I (-!)

Sph I OK

Sca I OK

Sma I OK

pV3000	phelper	pV3000	phelper	pV3000	phelper	pV3000	phelper
9767		11425	4425	12435	5435	7553	5183
3735	3735	2077	2077	1067	1067	4629	1319
	2767					1319	1319

no cleavage in
either pV3000
or phelper !!!

(or just one
site)

Sph I
OK

Xba I
OK

Sma I
OK

OK



Sph I

Xba I

Sma I

1 kb

8/3/94

Wednesday

No 5, 6, 7 and pV3000

cleavage

w/ Nco I

Nco I

pV3000	phelper
6451	6500
3910	
2051	
1090	



Sum many: according to four categories (No.1, No.2, No.3, No.4) we are ~~not~~ respond to doctor we are looking for Mike says that No.1 is missing in some of his

70

6500	0601
phlyper	1202
	03910
	1579
	0000

Nco L

W/N 201

№ 5, 6, 7 and 8

Wednesday
8/3/94

156 I 157 I 158 I 159 I

1000

5183
+ 319

1319

4290

LEGO

74

173



2

Genes 4/12 transcription

①

P2

P2 sure

70 µl

per 20 µl

4.1 gation mix

P1-5

is inoculated into 1 ml of LB + 0.5% yeast growth for 10 hrs, inoculated into 200 ml of LB + 0.5% yeast growth o/n for preparative DNA isolation (for transcription of RNA)

8/4/1994
Thursday

1. PV3000 inoculated (about 7 colonies) from the plate into 1 ml of LB + 0.5% yeast growth at 37°C for 4 hrs, inoculated into 200 ml of LB + 0.5% yeast growth o/n at 37°C
2. Wizard (Prosecco) helper

1. Wizard Maxi
 2. Cleave Max (NOT I) 9.20 - 12.00
- PV3000 (100 µl) P1-5 w/ NOT I (1.5 ml) preparative treatment phenol treatment precipitation wash twice w/ H₂O

1. 1 μ l of P1-S cleared
2. 5 μ l — — —
3. 15 μ l — — —
4. 10 μ l of purified
5. 1 kb

The amount of p...
 $\sim 100 \text{ ng}/\mu\text{l}$

Assuming the amount of p...
 the same level, the P1-S...
 plasmids are cleared with Not I

100 μ l of DNA	100 μ l of DNA
150 μ l of 10x R	50 μ l of 10x R ③
1250 μ l of water	300 μ l of water
μ l Not I	μ l Not I

$\Sigma 1500 \mu\text{l}$ $\Sigma 1500 \mu\text{l}$
 $\Sigma 500 \mu\text{l}$ $\Sigma 500 \mu\text{l}$

1. 10 μ l of P1-S/Not I + 3 μ l dye } diluted DNA
2. 10 μ l of p3000/Not I + 3 μ l dye }
3. 10 μ l of P1-S/Not I + 3 μ l dye }
4. 10 μ l of p3000/Not I + 3 μ l dye }
5. 1 kb ladder

Resumed:
 Cleavage complete
 + 1 vol isopropanol + NaCl
 left -20°C o/n

2/6/1994
 Saturday

Precipitates are washed once with 75% EtOH,
 dried, dissolved in water and combined,
 total volume 200 μ l. treated with
 phenol-chloroform + NaCl + EtOH 15' at -20°C ,
 spin down, wash twice, dissolve in
 15 μ l of water

TRANSCRIPTION REACTION

2500 units
Cat #8050 Ambion

	Water	10X dNTP	NTP/GTP	GTP	CAP	³ H ATP	RNA	Pol	
(630 μl) 5	3	-	9	3	1	1	5	3	mike's
to 20 μl	2	no 2 μl	75 mM T7	-	-	1	2	2 (1 μg)	Megascript M
9	3	12	50 mM SP6	-	-	1	2	3 (1.5 μg)	no cap

60 μl Promega's 5x
Ambion 2130 Waco and St #200
Ambion Texas 78222 - 1832
1000-88-204 phone

with cap
I

w/out cap
II
mG(5') PAP(5') G

1. Lassa LSA 3 μl
2. Mhelp RSR I 4 μl
3. PI-5 1.5 μl
4. V3000 1.5 μl

polymerase 2.5 μl (with cap) + 2.0 μl (w/o cap)

inh II
(w/o cap) Promega 5x 6 μl

37°C (in incubator) 2 hrs → -20°C (done!)

BHK D-29 cells (~90% grown)

discard media

add 5ml Trypsin-EDTA, rinse with

discard (after 2-5 sec of rinsing)

add 3ml of Trypsin-EDTA, rinse until
cells float (~5 min);

add 5ml of EMEM

dispense into flasks (2ml into
each) incubate +37°C

humidity 99%

CO₂ 5%



8/7/1994

Sunday

1. Cells are ~90% confluent
(for transformation is necessary
80-90%) => grown 0% at 37°C

2. estimation in transcription

1) the denaturing gel

1. GsaA replicon/cap

2. RsaI

3. P1-5

4. pV3000

5. GsaA

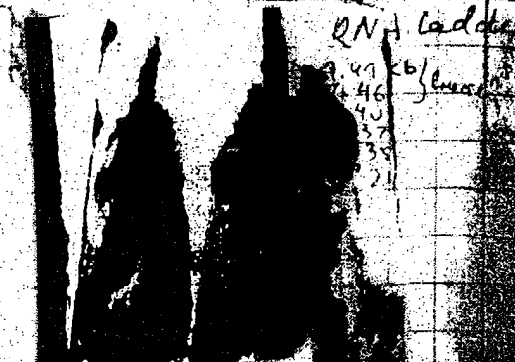
6. RsaI

7. P1-5

8. pV3000

RNA marker

replicon/cap



Amber cap¹ (39 mM)

DNA

water
H₂O
volume

5 X
25 mM NTPs
DNA

Enzyme
water
H₃-ATP

2 μ l
(4 μ l)
4 μ l
40 μ l
11 | 10 | 8

A 014

uncapped

8
12 my 3 lassa Rsr
② ③ ⑤
4
13 12 10
40 μ l

RNA gel

- ① lassa capped (as in 8/7/1994)
- ② Rsr1 capped
- ③ PI-5 capped
- ④ PV3000 capped

LSV cap
PI-5
LSV cap
PI-5
LSV cap
PI-5
LSV cap
PI-5



LSV RSD
lassa RSD
capped capped

2 μ l of Proneva's
Ribomax

3 μ l 3 μ l 3 μ l 3 μ l
PI-5 LSV RSD PI-5 V3000
capped cap cap cap
from 8/7/1994

Transfection of Bulk cells

Promega's RNAs	1.	pv3000 uncapped (as PC)	10 μ l (concn. see below)
	2.	LSN capped (as NC)	10 μ l
	3.	LSN - / -	{ 10 μ l }
		Rsr - / -	{ 10 μ l }
	4.	LSN - / -	{ 10 μ l }
		PI-5 - / -	{ 20 μ l }
	5.	PI-5 - / - (as NC)	{ 20 μ l }
	6.	Rsr - / - (as NC)	{ 10 μ l }

Promega's
 LSN 200 ng/ μ l cap + 10 μ l
 Rsr 200 ng/ μ l cap + 10 μ l
 PI-5 100 ng/ μ l cap + 20 μ l
 pv3000 200 ng/ μ l uncapped 10 μ l

mistake
occurred

Ambion's
 LSN 200 ng/ μ l cap + 10 μ l
 Rsr 200 ng/ μ l cap + 20 μ l
 PI-5 200 ng/ μ l cap + 20 μ l

7.
~~LSN~~ LSN capped { 10 μ l }
 Rsr - / - { 20 μ l }
 8. LSN capped { 10 μ l }
 PI-5 - / - { 20 μ l }

8/9/94
Tuesday

transcriptions (Promega's Ribomax)
 25mM each
 100mM
 39mM
 Polymerase

5 (~3mM each)
 0.3 (~0.5mM)
 2 (~2mM)
 2
 4

total vol.

40.3 μ l

⇒ small amount of RNA
 but probably a lot of capped!

Immunofluorescence

cells grown in the special vial of
 grow medium removed
 cells washed twice in ice-cold PBS 2x 5min
 cells dried
 cells soaked in acetone for 10 min
 dried

incubated with antibody load ab on a thin
 layer

Lassa ~ 120 μ l undiluted

VEE dilution 1:100 in 20% FBS (PBS)

wash in PBS 20 min stirring and change in PBS

dry plates, Lassa are ready for microscope

VEE second a-mouse fluorescein labeled anti-nucleoprotein
 ToG (R+1) (anti-H1)

Cap 100 mM
 RNA 39 mM
 Polymerase

2 (~ 2 mM)
 2
 4

total vol.

40.3 μ l



⇒ small amount of RNA
 but probably a lot of capped

Immunofluorescence

cells grown in the special vial of
 grow medium removed
 cells washed twice in ice-cold PBS 2x 5 min
 cells dried
 cells soaked in acetone for 10 min
 dried

incubated with antibody load ab as a thick layer

Lassa ~ 120 μ l undiluted

VEE dilution 1:100 in 20% FBS (PBS)

wash in PBS 20 min stirring and change in PBS

dry plates, Lassa are ready for microscope

VEE second a-mouse fluorescein labeled anti-mouse
 IgG (H+L) (coat) human
 diluted 1:75 serum adsorbed

8/10/1994
Wednesday

Transcriptibus (R. Lomas)

water	14
SX	8
MTF - G 25 mM each	10
G 100 mM	0.5
cap 39 mM	2
dNA	2
polymurase	4
	40.5 μ l

G 100 mM 0.5
 Cap 39 mM 2
 DNA 2 (or 1 μ l for P1-5 and V3000 (+ 1 μ l water))
 polymerase 4
 40.5 μ l



Amount of replicon VLPs

1. Trypsinize roller bottle with 50 μ l of Trypsin-EDTA ~~at RT~~ ~5' at RT (~20°C)
2. Remove Trypsin-EDTA
3. Add 10 ml of Trypsin EDTA increase ~6' at + RT (~20°C) until cell just begin to come out of the surface (+ 5 μ l of fresh Trypsin ~3 min at RT)
4. Discard Trypsin EDTA
5. Add 10 ml EMEM, suspend to homogeneity by aspiration (~15 ml)
6. 2 ml into large flask + 40 ml of EMEM
7. Count the cells
8. 100,000 cells / per one cell (0.4 ml)

dilutions
 1 2 3 4 5 6 7 8
 V 3000 1:100 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻⁶ 10⁻⁷ 10⁻⁸ 10⁻⁹
 (1:100) (10⁻³) (10⁻⁴) ~~10⁻⁵~~
 (SN + RSR) 10⁻² 10⁻³ etc

10⁻²

10⁻²

10⁻²

10⁻²

10⁻²

10⁻²

10⁻²

10⁻²

morphology of cells

slowly grow, rounded cells
 quick grow, filaments
 quick grow, — —
 quick grow, — —

RSR
 + PI-5

LSN + PI-5

slowly grow, rounded cells, as in ①

Calculation of cells

~ 20 cells per smallest square

↓
 $2 \cdot 10^5$ cells/ml

I need to seed ~ 100,000 cells per chamber

↓
 400 µl into each chamber

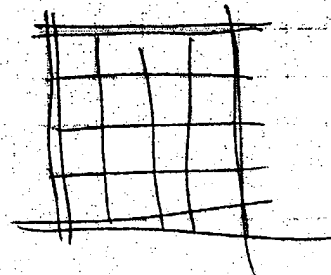


Plate	Dilutions / chambers							
	1	2	3	4	5	6	7	8
1. V3000	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
2. LSN/RSR	—	—	—	—	—	—	—	—
3. LSN/PI-5	"	"	"	"	"	"	"	"
5. LSN	"	"	"	—	—	—	—	—

cell 8/11/94
 Thursday

V3000	LSN	LSN RSR	LSN PI-5
undiluted	un-	un-	un-
10^{-3}	10^{-3}	10^{-3}	10^{-3}

IFA

1. Plates (Lab-Tek)
Chambers and rubbers are removed,
plates washed 3x5' in PBS
(fairly cold)
2. Plates are dried on the bench
3. Plates are fixed in cold acetone
4. Plates are dried
5. Incubation with new Cassa # 288 AGS
Conjugate (plates 1, 2, 3, 5) or
mouse a-VEE AG (plate 4)
30 min
6. Wash in ice cold PBS 2x10 min on
magnetic stirrer
7. Dry plates, (also ready for microscop)
a-VEE (plate 4)
100 μ l of a-mouse conjugate
1:75 in 20% FBS in PBS
= 0.5(?)
8. Plate 4 washed 20 min w/PBS
dried
+ mounting media $\rightarrow$ microscope

a - Cassa

- ① pV3000 all negative
- ② LSN/RSL
3 cells positive in 10⁻² dilution
- ③ LSN/PI-5
2 cells positive in 10⁻² dilution
- ⑤ LSN all negative

a VEE

- ④ pV3000 - negative
- LSN/RSL - negative
- LSN - negative
- LSN/PI-5 - positive

cells are splitted

8/15/1994
Monday

description, (protocol from 8/10/1994)

water 14

5x 8

NTP-G 10

G 0.5

cap 2

DNA 2

pol 4

(pV3000 : 1 μ l of Amax +
2 μ l Pharmacia 20 mM)
(1 μ l P and V)

Bank cells: 1) split and 2) electroporated (90-100% confluent
have clumps 70%-John K)

Questions: 1) are clumped cells become
normal monolayer after splitting?
... cells be used

EMEM/NEAA

PUSHKO

on:

0.5 ml

1.0 ml

+10% Fetal

(20 μ l)

(20 μ l)

Bovine

Serum

(20 μ l)

Not enough cells

for electroporation

200 ml - flask

has about 1×10^6 cells

I need 1×10^7 per ml

and 500 μ l per electropo

Bank cells are 40-50% confluent

8/16/1994
Tuesday

Cells are ~80-90% confluent (2 x 540 ml flask?)

8/17/1994
Wednesday

Electroporation BUK 2-1 with RNAs

Samples:

- V. ① μ 3000
 R. ② LSN/RSE
 P. ③ LSN/P1-5
 - ④ - (cells)
 cells ⑤ cells w/o electroporation

for electroporation
2 x 540 μ l flasks is
just enough

(~1 μ g!) Cells were counted $\sim 6 \times 10^4$ / ml (Σ 40 ml) $\Rightarrow 2.4 \times 10^7$ cells
 4 μ g of RNA Rost suspended in 100 μ l of ice-cold PBS
 were used for each electroporation $\sim 1.0 \times 10^7$ cells/ml (as in protocol)

Time const Electroporation, as described in protocol

= 0.8 (!) Electroporated cells were seeded into either

LABTEK cover slips or small flasks at 10:30 am

② or ⑤ antibody

✓	V	R	P	-
✓	V	R	P	cells

400 μ l / chamber or 100 μ l / plate
 for tomorrow morning
 or 400 μ l / chamber for
 analysis at 4:30 pm (6 hr post exp)

Remaining cultures - into flasks
 and grow 24 hrs (10:30 -

BUK cells ($\sim 70\%$ confluent 1 x 540 μ l / flask)
 trypsinized
 cells counted = $5-6 \times 10^4$ / ml

$\sim 20-25$ no. of cells per chamber of LABTEK slips

1L of 10x PBS

80g NaCl

2g KCl

11.5g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (14.4g Na_2HPO_4)

2g KH_2PO_4

(125g $\text{K}_2\text{H}_2\text{PO}_4$) 0.95g K_2HPO_4

Cells Vs and Ls were taken after 6 hrs incubation
 IFA was done as described in protocol, with anti-Lassa
 and anti-VEE antibodies

Simultaneously, after 6 hrs p.e. (post electroporation)
 supernatants were taken and
 stored at -70°C (From flasks)

Result: 1) less than 1% of cells were positive for Lassa antigen after 6 hrs p.e. 5-10 cells per view
 2) roughly 1% are positive for VEE antigen 10-20 cells per view

IFA of cells electroporated 8/17/94

8/18/94
 Thursday

q-Lassa: 30 hrs p.e. ~ 1 (max 10)% of cells express Lassa antigen, not pV3000 or NC.

q-VEE: 30 hrs p.e. ~ 1 (max 10)% of cells express VEE antigen / Rst & PI-5 helpers, pV3000 (cells); no VEE antigen in (-) controls / electroporated w/o RNA cells

Electroporation efficacy is low (1-10%)

Supernatants were taken from transfected cells 6 and 30 hrs p.e., diluted 10^{-1} , 10^{-3} , 10^{-5} , 10^{-7}

	10^{-1}	10^{-3}	10^{-5}	10^{-7}
	-1	-3	-5	-7
	10	10	10	10

100 μl of sup + 900 μl EMEM
 10 990
 10 990
 10 990

NC	-1	-3	-5	-7
	-7			

400 μl of such mixtures were put to chambers of 8-chamber (Lab Tek plates (Nunc)) incubated 18 hr (O/N)

8/19/94
 Friday

IFA for Lassa antigen of cells infected with repl con containing VLPs (RVLR)

V₆p.e. = "0 cells"; V₃₀p.e. = "0" at all dilutions (10^{-1} , 10^{-3} , 10^{-5} , 10^{-7})

for Lassa antigen after 6 hrs p.e. per vial
 2) roughly 1% are positive for
 VEE antigen

A 025

8/18/94
 Thursday

IFA of cells electroporated 8/17/1994

q - Lassa : 30 hrs p.e. ~ 1 (max 10)% of cells express
 Lassa antigen, not pV3000 or NC.
 q - VEE : 30 hrs p.e. ~ 1 (max 10)% of cells express
 VEE antigen / Rst & PI-5 helpers
 pV3000 (cells); no VEE antigen in
 (-) controls / electroporated w/o RNA cells

Electroporation efficacy is
 low (1-10%)

Supernatants were taken from transfected
 cells 6 and 30 hrs p.e., diluted
 10^{-1} , 10^{-3} , 10^{-5} , 10^{-7}

100 μ l of sup + 900 μ l EMEM
 10 990
 10 990
 10 990

10^{-1}	10^{-3}	10^{-5}	10^{-7}
-1	-3	-5	-7
10	10	10	10

NC	-1	-3	-5	-7

400 μ l of such mixtures
 were put to chambers of
 8-chamber (al. Titer plates (Nunc))
 incubated 18 hr (O/N)

8/19/94
 Friday

IFA for Lassa antigen of cells infected
 with replicon containing VLAs (RVLR)

$V_{6p.e.}$ = "0 cells"; $V_{30p.e.}$ = "0" at all dilutions (10^{-1} , 10^{-3} , 10^{-5} , 10^{-7})
 $R_{6p.e.}$ = "0"; $R_{30p.e.}$ = "3" at 10^{-1} dilution; "0" at other dilutions
 $P_{6p.e.}$ = "0"; $P_{30p.e.}$ = "10" at 10^{-1} dilution; "0" at other dilutions
 $\bar{c}_{30p.e.}$ = "0"; $\bar{c}_{6p.e.}$ = "nt" at all dilutions

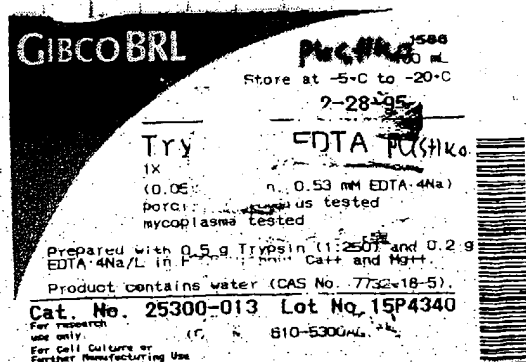
(3×10^5) versus (5×10^6)

3×10^5 ~~клеток~~
расщеплен 6500 пп $\Rightarrow$
 6×10^5 / ml

Time constant 0.7 for 2.5 kV - $\tau_{max} \sim 400 \mu s$
0.9 for 1.5 kV - $\tau_{max} \sim 350 \mu s$

9.5 ml EMEM + 0.45 ml electroporated cells

2 of 8-chance
Lab Tex



LIFE TECHNOLOGIES

TEF-7 cells, electroporated 8/19

8/22/1994
Saturday

cells were electroporated w/ pV3000 RNA $\Rightarrow$
all must be positive since virus produced
by transfected cell is infectious and
should infect untransfected cells.

According to protocol, with a-VEE
alteration of protocol: after first AB
slides washed 2 times w/PBS
(according to protocol, should be 3 times)
after conjugate
slides washed 3 times w/PBS
(according to protocol, should be 3 times)

In both cases (e.g. with either 2.5 kV or 1.5 kV)
VEE-positive cells constitute $\sim 5\%$ of total
cell population (no difference in
transfection efficacy for 2.5 kV and 1.5 kV)

May be, they became "equal"
(same percentage of VEE-positive cells)
after transfection, due to infection
with produced virus?

Transcription Ambion in Message in Machine

8/21/94
Sunday

(50)(55)	U2	(LSN)	(p1-5)
20 μ l	10X Buffer		
100	2X		
10(5)	DNA LSN(p1-5)		
20	10X Enz		

200



Cells infected w/ PV300 RNA and
supposed to produce infectious
virus seem to grow well,
without visible alterations? may be,
cells e.p. at 2.5 kV are not filamentous,
rather round-shaped, with "pili"

BHK cells are splitted for tomorrow

8/22/1994

Monday

Electroporation

7/15/80 BHK cells are grown to ~80-90%
density $2.7 \cdot 10^7$ cells/ml
Suspended in 2.5 ml $\Rightarrow 1 \cdot 10^7$ cells/ml

Samples:

- ① 1.5 kV ; as in protocol
 - ② 1.5 kV ; 37°C after e.p. (10')
 - ③ 1.5 kV ; + EMEM (37°C), 10' at RT
 - ④ 1.5 kV ; 5 times ; 10' RT
 - ⑤ 2.5 kV ; 2 times ; 10' RT
 - ⑥ cells, no RNA, 2.5 kV 5 times ; 10' RT
- 20 μ l LSN RNA
+ 20 μ l H₂O
+ 450 μ l KAESON

a-lassa

microsome
no supernatant

microsome
supernatant
KESON
or microsome
supernatant
KESON

- 10/40 incubation at 37°C started (6 hrs)
- ① let 100% at 0% ~30% of live cells are positive
 - ② ~100% ~40-50% are positive
 - ③ ~100% ~5% are positive
 - ④ ~10% a lot of dead
 - ⑤ ~10% dead
 - ⑥ ~1% all cells are dead

if ① = 100% of cells
(suppose)

if the cells attached
to the bottom
④⑤⑥ show only
10% in comparison
to ①②③

26/200 18%
31/140 22%
positive/total cells

IFA

we found
no ye now
no bee
now

microsome
supernatant
KESON
no microsome
supernatant
KESON
microsome
supernatant
KESON
microsome
supernatant
KESON
microsome
supernatant
KESON

after
at 37°C
11 hrs
11/40

cells
OTK
or
slide

rather round-shaped, with fine

A 029

BHK cells are splitted for tomorrow

5/22/1994

Monday

Electroporation

7.150 BHK cells are grown to ~80-90%
cells $2.7 \cdot 10^7$ cells/ml
Suspended in 2.5 ml $\Rightarrow 1 \cdot 10^7$ cells/ml

Samples:

- ① 1.5 kV; as in protocol
 - ② 1.5 kV; 37°C after e.p. (10')
 - ③ 1.5 kV; + EMEM (37°C), 10' RT
 - ④ 1.5 kV; 5 times; 10' RT
 - ⑤ 2.5 kV; 2 times; 10' RT
 - ⑥ cells, no RNA, 2.5 kV 5 times; 10' RT
- 20 μ l CSN RNA
+ 20 μ l H₂O
+ 450 μ l Kassa

a-lassa

no response
or response

1040

incubation at 37°C started
(6 hrs)

2140

(11 hrs)

- 100% IFA positive
- ① ~100% ~30% of live cells are positive
 - ② ~100% ~40-50% are positive
 - ③ ~100% ~5% are positive
 - ④ ~10% a lot of dead
 - ⑤ ~10% - dead -
 - ⑥ ~1% all cells are dead
- many cells are unresponsive
or many cells are responsive

many cells are unresponsive
no response
response ~ 50%
cells are unresponsive
cells are responsive

if ① = 100% of cells
(suppose)

if the cells attached
to the bottom
④⑤⑥ has only
~10% in comparison
to ①②③

many unattached
cells in all samples

36/200 18%
31/140 22%
positive/total cells

IFA
we failed
we see
no cells
at bottom

B. Z. Zuckerman et al., January 5, 1993

Sindbis virus vectors

A 030

Protocol for electroporation of BHK-21 cells:

Reference: Liljeström et al., J. Virol. 65:4107-4113 (1991).

Cells: BHK-21, low passage number from ATCC. Culture medium: MEM+NEAA+10% FBS or DMEM + 7.5% FBS. When passaging or preparing cells for electroporation, BHK monolayers should never be allowed reach complete confluence. This causes clumping and is generally irreversible. If this happens, throw them away and start over. Note that you will need 5×10^6 cells per electroporation, use cells that are 60-80% confluent.

Apparatus: BioRad Gene Pulsar™ with Pulse Controller. 0.2 mm electrode gap cuvettes from BioRad. Other brands of electroporation devices probably will also work but may require adjustment of settings (voltage, capacitance, etc.) to optimize transfection efficiency and cell survival.

RNase-free PBS: Cells are washed and shocked in this solution. It is advisable to check the electroporation time constant of your PBS before starting, the value should be 0.7 at the settings given below.

1. Wash the cell monolayers twice with PBS.
2. Add 3 ml trypsin per 150 mm plate. Incubate at 37°C for ~ 5 min until trypsinization is complete. Gently produce a single cell suspension in the trypsin solution using a pipette and transfer the cells to MEM + 2% FBS to stop trypsinization (10 ml per plate).
3. Collect the cells by low speed centrifugation at room temperature. *500 rpm, 5 min*
4. Carefully remove the supernatant and resuspend the cells in ice-cold RNase-free PBS (we use 3-5 ml per 150 mm plate). *10 ml*
5. Determine the yield of the cells (we usually count the number of cells diluted 1:10 in PBS).
6. Collect the cells as in step 3. Wash one additional time with ice-cold RNase-free PBS (repeat steps 4 and 3).
7. Resuspend the cells at a concentration of 1×10^7 cells/ml in ice cold RNase-free PBS and keep them on ice.
8. Put the RNA samples to be electroporated in a 1.5 ml microcentrifuge tube on ice. Add 500 µl of the cell suspension, mix gently, and quickly transfer the RNA/cell mixture to the electroporation cuvette. Put the cuvette in the holder of the electroporation device.
9. Shock the cells two times using the following settings. 1.5 kV, 25 µFD, with the pulse controller resistance set on infinity. The time constant should be approximately 0.7. Cell survival is typically 65-75%.
10. After the electric shocks, allow the cells to sit at room temperature for 10 min. After this recovery period, the cells are diluted 20-fold with MEM + FBS (9.5 ml) and plated. We usually use 0.5-2 ml/ 24 mm well, 1-4 ml/ 35 mm plate, 4-6 ml/ 60 mm plate, or 8-10 ml/ 100 mm plate, depending upon nature of the experiment.

2 cm

Make - most recent procedure from Bob's lab. Looks like it's the same as before - it probably should be.

4 hrs p.e. 10 μ l of supernatant was taken and frozen on
The slides were subjected to IFA w/ α -VEE.
~1-2% positive

6 hrs p.e. α -VEE and α -Lassa
~30% positive ~20% (+)

8 hrs p.e. α -VEE (2X)
not clear, there are positive cells: either all positive or only 1-2% positive

10 hrs p.e. washed, dried, acetone treated (10'), dried, ~~fixed~~
 α -VEE α -Lassa
not clear ~20% positive

8/26/1994
Friday

24 hrs p.e. α -VEE
not clear, too many cells,
which probably make antibodies
within the cells inaccessible to antibodies

BHK cells are growing for
electroporation (~40% confluent)

8/28/1994
Sunday

Electroporation

- ① 25 μ l CSN + 25 μ l PI-S as in protocol
 - ② $1-3 \cdot 10^7$ cells before electroporation 10-75 kV and 10' a
- 10' 40' -

BHK seeded on 6 slides at
Quebec conc. $1.2 \cdot 10^5$ cells per chamber at 8-10' (24 hr)

6 hrs p/e α -VEE
① ~25% positive
② ~20% positive

8 hrs p.e. a-VEE (2x)
not clear, there are positive cells: ^{either a} positive only ^{only 10%}

10 hrs p.e. washed, dried, acetone treated (10'), dried, ~~10'~~
a-VEE a-lassa
not clear ~ 20% positive

8/26/1994
Friday

24 hrs p.e. a-VEE
not clear, too many cells,
which probably make antigens
within the cells accessible to antibodies

BHK cells are growing for
electroporation (~40% confluent)

8/28/1994
Sunday

Electroporation

- ① 25 ml CSN + 25 ml DM-5 as in protocol
② 1-3 $\cdot 10^7$ cells before electroporation 10.75 kV and 10'.

BHK seeded on glass slides at
Queens house. $1.2 \cdot 10^5$ cells ~~per~~ per chamber of 8-
(cont)

6 hrs p/e a-VEE
① ~ 25% positive
② ~ 25% positive

a-lassa
① ~ 15% of cells are positive
② (1.75 kV; 10' at 37°C) ~ 10% of cells are positive

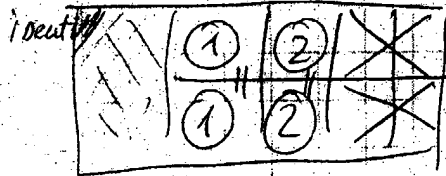
supernatants are frozen (10 μ l and 1.5 ml)
 aliquots
 Slides are washed w/PBS 10' and dried,
 kept o/n at -20°C.

A 033

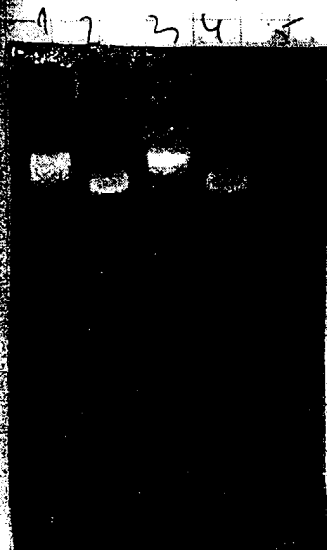
8/29/94
 Monday

hrs p.e. w/o o/n freezing (of course)

IFA w/ α -VEE and α -Lassa



Plasmid DNA cleavage w/ Not I



- ① LSN
- ② BSR
- ③ pV3000
- ④ PI-S
- ⑤ K6 ladder

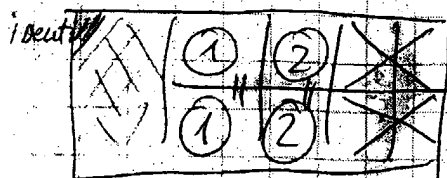
125 μ l/well (w/ 400 μ l)

α -Lassa

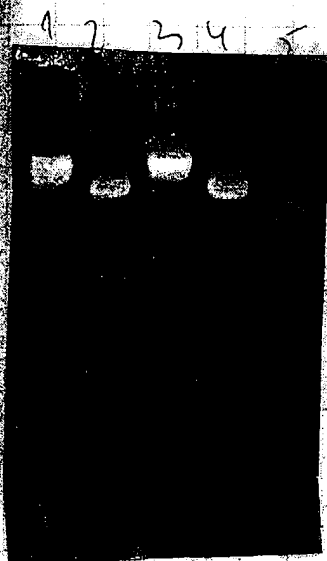
p.e., hrs	number of positive cells (mean)	total no. of cells (mean)	positive cells (percent)
6	1) 60, 40; (50) 2) 39, 20; (29.5)	ND ND	ND ND
9	1) 85, 83, 60, 68 (74) 2) 67, 70, 43, 60 (55)	ND 500	ND 12%
12	1) 76, 88, 80 (81) 2) 90, 46 (68)	ND 500, 400	ND 18% 11.5%

22 hrs p.e. w/o of freezing (of course)

IFA w/ α -VEE and α -Lassa



Plasmid DNA cleavage w/ Not I



- ① LSN
- ② PSR
- ③ pV3000
- ④ pI-5
- ⑤ K6 ladder

100 μ l/well (w/ 400 μ l)

α -Lassa

p.e., hrs	number of positive cells (mean)	total no. of cells (mean)	positive cells (percent)
6	1) 60; 40; (50) 2) 39; 20; (29.5)	ND ND	ND ND
9	1) 85; 83; 60; 68 (74) 2) 67; 50; 43; 60 (55)	ND 500	ND 12%
12	1) 76; 88; 80 (81) 2) 90; 46 (68)	ND 500; 400	ND 18%; 11.5%
22	1) 70; 65; 52 (62.3) 2) 100; 58 (79)	600 600; 200	8.7% 16.6%; 29%

20

p.e. hrs	Q-VEE		total no. of cells	positive cells (percent)
6	1)	39; 45 (42)	ND	ND
	2)	36; 38 (37)	ND	ND
9	1)	35; 7 (21)	ND	ND
	2)	19; occasional	200	9.5%
12	1)	18; 14 (16)	250	5.6%
	2)	10; occasional	~~250~~ ND	ND
22	1)	23; occasional	ND	ND
	2)	10; occasional	~~250~~ 400	2%

40x / 0.75 occasional
10x / 25 occasional

cells were rare

8/29/94
Tuesday

RNA's : transcription of LSN
Rsr
Vse
P1-5

Preparations:	uncapped	capped	capped
Promega kit	lmo 10	water	~~19~~ 23
LSN	5x 8	5x	16
transcription	NTP 10	NTP-G 10	20
	RNA 4(?)	G 0.5	1
	pol 4	Cap 2	8 (20mM)
		RNA	~~4~~ 4
	40µl	pol 9	8
		40µl	80µl

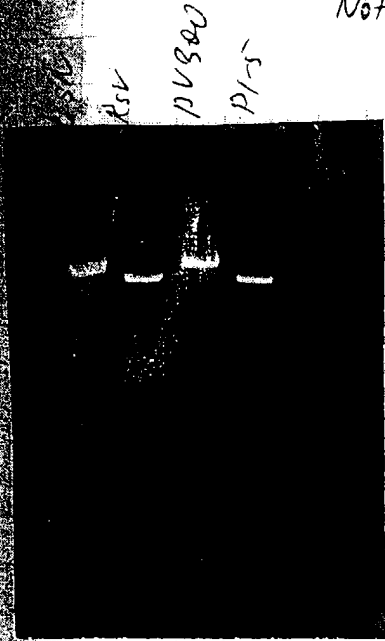
m³A(5')ppp(5')G
27-4635-02 25 u

1.4 317-5021
S. H. G. C.
For the latest price list
Made in USA



infection with replicon p
Media from BHK cells
removed by vacuum
100 μ l of 10^{-2} d
transfected cells
Media added (300 μ l)
continued at 37°C

Cleaved DNA
Not I



6 hrs post infection		1.5 kV; 2 hrs; 10°C RT	
3	51	104	
6 hrs p.e.	9 hrs p.e.	12 hrs p.e.	22 hrs p.e.
	1.75 kV; 2 hrs; 10°C		
0	43	123	
electroporated amount of positive cells/well = particles/ml			
1.5 kV; 2x; 10°C RT			
ND	ND	34	88
4 hrs p.e.	6 hrs p.e.	10 hrs p.e.	22 hrs p.e.
ND	ND	66	127
1.5 kV; 2x; 10°C RT			

1 μ l (SN) cap
3 μ l (LSN) cap
3 μ l (LSN) cap
3 μ l (PT-100) cap
100 μ l (100) 100 μ l (100) 100 μ l (100) 100 μ l (100)



total
4 μ g 3 μ g 3 μ g

A 036

see next page

from capped RNA (80 μ l) 40 μ l has been taken
+ 20 μ l LiCl Precip. soln
at least 30 min - 20°C (2 hrs in reality)
15 min at max rpm.
wash 75% EtOH
dry, dissolve in 30 μ l water

left to right:

- ① LSN uncapped w/DNA (2x)
- ② - - - capped w/DNA (1x)
- ③ - - - - - w/o DNA
- ④ P1-5 capped/ambion

1 μ l of eachLiCl
removed

8/31/1994

IFA of infected suk cells : a-lissa
 infected with replicon LSN + P1-5 helper, 24 hrs p.i.
 (post-infection)

④ dilution 10^{-2}	ND	ND	MP	488	} 1.5kV; 2x; 10' RT
	6 hrs p.e.	2 hrs	12 hrs	22 hrs	
	ND	ND	134	529	} 1.75kV; 2x; 10' 37'

24 hrs post infection

10^9 cells/ml for electroporation

24 hrs post infection

see 6 hrs post infection
on a previous page

10^9 cell/ml for electroporation

④ dilution 10^{-2}	~~ND~~	6 hrs p.e.	10 hrs p.e.	24 hrs p.e.	} 1.5kV; 2x; 10' RT
	~~ND~~	6 hrs	10 hrs	24 hrs	
	~~ND~~	ND	193	475	} 1.5kV; 2x; 10' 37'
	~~ND~~	ND	228	279	

Electroporation

- 10 μ l of LSN uncapped (Fig. p. 28, lane 1) + 5 μ l H₂O;
- 10 μ l of capped RNA with DNA and nucleotides (lane 2)
- 10 μ l of capped purified RNA (lane 3)

- 15 μ l of lane ① + 16 μ l H₂O
- 5 μ l of lane ② + 20 μ l H₂O
- 35 μ l of lane ②
- 3 μ l of lane ③ + 20 μ l H₂O
- 25 μ l of lane ③
- 15 μ l of lane ① at 37° 10

Time 0.3!?

11% efficiency (5-10%)
 25-30% (40%)
 18-21% (50-60%)

5-6%
 5-35%

Time const 0.3?! (~10%)

Makeup, up over
 H₂O + 0.8 = 0.3!?

incubation at 37° 10 $\div$ 7³⁰ (~13 hrs)
 started

IFA with α -laser conjugate

9/1/89
 Thursday

Transcriptions

	① LSN - Promega	② PI-5 - Promega	③ LSN - Antibio	④ PI-5 - Antibio
water	23	23	23	23
5x	16	16	16	16
ATP-G	20	20	20	20
G 100mM	0.5	0.5		
cap 20mM	8	8		
DNA	4 (LSN)	4 (PI-5)		
pH	8	8		
Σ	80 μ l	80 μ l	80 μ l	80 μ l

(as described in manual)

40 μ l + 20 μ l cell 30' at -20°C, cf, wash, dry, dissolve in 3

15mM each C, U, A

~~100 µl of capped RNA with DNA and nucleotides (lane 2)
 100 µl of capped purified RNA (lane 3)~~

A 039

50 µl of lane ① + H₂O
 50 µl of lane ② + 20 mM
 35 µl of lane ②
 35 µl of lane ③ + 20 mM
 25 µl of lane ③
 15 µl of lane ① at 37° 10'

Time 0.3?!

11% efficiency (5-10%)
 25-30% (40%)
 18-21% (30-40%)

5-6%
 50-55%

Time cost 0.3?!

H₂O + H₂O
 H₂O + H₂O
 0.8 = 0.3?!

incubation at 37° 10' ÷ 7³⁰ (~13 hrs)
started

IFA with α-lisa conjugate

9/11
Thurs

Transcriptions

① LSN - Proomega

② PI-5 - Proomega

③

③ LSN - Ambion

④ PI-5 - Ambion

23
 16
 20
 0.5
 8
 4 (LSN)
 8
 Σ 80 µl

23
 16
 20
 0.5
 8
 4 (PI-5)
 8
 Σ 80 µl

23
16
20

Σ 80 µl
 Σ 80 µl
 (as described in manual)

40 µl + 20 µl lill 30' at -20°C, wash, dry, dissolve

15 mM each C, U, A
 0.6 mM G
 2 mM Cap

9/2/1997

Friday

RNA gel

① ② ③ ④ ⑤



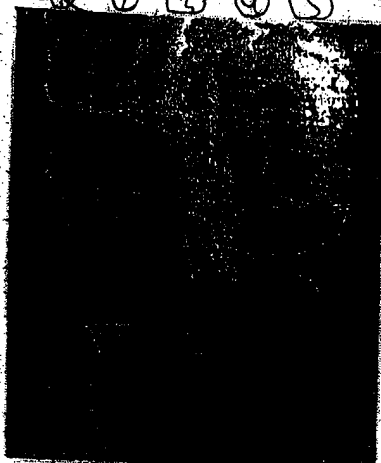
- 1 Ambion LSN Liel⁺
- 2 Ambion PI-5 Liel⁺
- 3 Promega LSN Liel⁺
- 4 Promega PI-5 Liel⁺
- 5 Promega PI-5 Liel⁻

minor (~20 ug) band of RNA
 ④ degradation of RNA

IFA with 1) 4-6SSA { 12 hrs p.e
 2) 6-VEE } 18 hrs p.e

unpurified

① ② ③ ④ ⑤



- 1 Amb LSN Liel⁻
- 2 Amb PI-5 Liel⁻
- 3 Prom LSN Liel⁻
- 4 Prom PI-5 Liel⁻
- 5 Amb LSN Liel⁺

- 1 Amb LSN Liel⁺
- 2 Amb PI-5 Liel⁺
- 3 Prom LSN Liel⁺
- 4 Prom PI-5 Liel⁺
- 5 Amb LSN Liel⁻

150 ug/ml
 100 ug/ml
 20 ug/ml
 50 ug/ml

purified



400 ug 1 ug 2 ug 3 ug total
 50 ug/ml 100 ug/ml 150 ug/ml
 20 ug/ml 100 ug/ml

Does this
manuscript
mean the fusion
with vesicular
particles?

Electroporation 9/1/99

Samples

1. ① A UCE : Ambion 10µl LSN + 10µl P1-5, DNA⁻, cap⁺
 ② ② : — / — / — / — ; DNA⁺, cap⁺
 ③ ③ P UCE : Promega 10µl LSN + 10µl P1-5, DNA⁻, cap⁺
 4. ④ P : — / — / — ; DNA⁺, cap⁺

A 041

IFA 9/2/1994

12x78 hrs p.e.

~~Antibody~~

Conjugate

a-6959 #288

mean
12 hrs p.e.
% of positive
cells

mean
24 hrs p.e.
% of positive
cells

Antibody

a - VEF

Conjugate

2-mouse

~~Infection w/ supernatants from ab~~

~~Antibody~~

Conjugate

a-lassa

288

Construction

1) Искрившее, высоко мембранное РНА,
(~ 4 мк, ~~мемб~~ мемб. мемб.)

2) Плюс периметр двойки каковы
($\sim 10^7$ /мл), но в целом
выбраны чистые уменьшенные
фрагменты ~~фрагментов~~ фрагментов

DNA⁻, cap⁺
DNA⁺, cap⁺
-5; DNA⁻, cap⁻ 3/4 + РНА геотроп.
DNA⁺, cap⁺ 9) переносимости!!

1		2	
24/180	13	32/200	16
50/200	25	18/200	9
55/200	28	43/200	22
	22%	42/200	16
			16

3 0/0 0 4 0/150
3/43 7 0/200
10/200 5/ 3/200 2 1%
9 - 4% - Cassa, 18 hrs p. e

$a = V_{EE}$, 18 hrs p, 2

A

2

Agnes Jones

3

account

as to 12 hrs
p.e.

				Inserted

Inserted

between pg 30 & 31

RNA
TRANSFECTIONElectroporation 9/4/94, at room temp, 10' at 37°C p.e.

Samples

1. 30 μ l PI-5 (Amersham), unpurified
2. 20 μ l CSN (Amersham), purified
3. 10 μ l CSN (Amersham), unpurified
4. 60 μ l + ~450 μ l of cells ($\sim 1 \cdot 10^7$ /ml)
- 5.
- 6.
- 7.
- 8.

PBS washes:

IFA 9/5/94 5' before & after acetone; 10' after conjugate

Antibody

Conjugate

 α -lisse

#288

14 hrs p.e.	33/200 17% 24/170 14% positive cells / total no. of cells			
	31/300 10%			

mean of
positive cells

(14%)

PBS washes:

5' before & after acetone; 5' after Ab; 10' after conjug.

IFA 9/5/94

Antibody mouse

 α -VEE

Conjugate

 α -mouse

14 hrs p.e.	51/200 26% 22/200 11% 30/200 15%			

mean

(17%)

 $\Rightarrow$ only 850,000 cells (out of 5,000,000)
may produce VLPsInfection 9/5/94 (dilution
10⁻²)

① 14 hrs p.e. ② 16 hrs p.e.

IFA 9/6/94

hrs p.i.

Antibody

Conjugate

 α -lisse

#288

1) 14 hrs p.e. 2) 16 hrs p.e. young cells	① 0 / 1	② 46 / 3		
	① 4 / 0	② 12 / 4		

(~10²)
antibodies(10²-10³)
polyplexes

transformation

30 μ l of PI-5 Anion (8/21/94)
20 μ l VLSN/400 + 10 μ l Anion *

at RT; 10' at 37°C

9/4/94
Transfection
Sunday

IFA a-Lassa
a-VEE

(~14% efficiency)
17% efficiency

9/5/94
Monday
Saturday

BMK cells (young, 2 days; and old, 4 days)
infected w/ replication particles

IFA a-Lassa

of cells infected with replication particles

9/6/94
Tuesday

Transcriptions (in Message Machine)

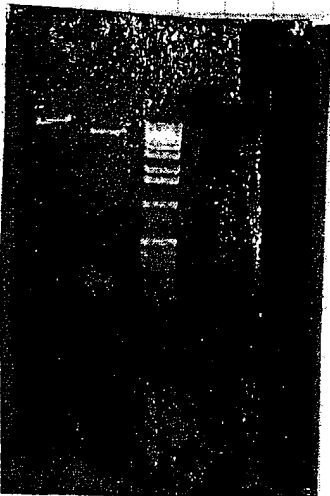
Water 20 μ l
10x buf 8
2x NTP/40
VLSN 4
10x Enz 8

80 μ l

~~Set~~ 17
10 8
40
PI-5 7
10 Enz 8

80 μ l

37°C 11' ±



replication (1) μ l non-replicating
(2) μ l

20µl of PI 5
 20µl VLSN/400 + 10µl Ambion *
 at RT; 10' 30"

A - Lassa (~14% efficiency)
 A - VEE 17% efficiency

cells (young, 2 days; and old, 4 days)
 infected w/ replication particles

FA A - Lassa
 cells infected with replication particles

Transcriptions (in Message Machine)

Water 20µl
 10x buf 8
 2x NTP/µl : 40
 VLSN 4
 10x Buz 8
 80µl

~~Water~~ 17
 10x 8
 40
 PI-5 7
 10x Buz 8
 80µl

37°C



lyse 1µl non-purified
 2µl

DNA gel ① VLSN
 ② PI-5 (Ambion, non-purified)

32

7/7/94

Wednesday

RNA LSN 9/6/94 5 μ lRNA PI-5 9/6/94 5 μ lRNA PI-5 8/21/94 5 μ lDNase I (24 μ l, Ambion
no MnCl₂)0.25 μ l0.25 μ l0.25 μ l

37°C 15'

37°C 15'

37°C 15'

(1.5 μ l) + 0.5 μ l + 0.5 μ l + 2.5 μ l + (1.5 μ l ETBR) + (+ 1.5 μ l BPR)
 RNA 10x PB Formamide Formalin

occlusion $\rightarrow$ -20°C for RNA gel

RNA gel:

DNA gel:



- | | | |
|----|--------------|----------------------------|
| 1. | LSN 9/6/94 | } T7 transcription mixture |
| 2. | PI-5 9/6/94 | |
| 3. | PI-5 8/21/94 | |

RNA gel	DNA
1.5 μ l of reaction	3.5 μ l
1.5 - - -	3.5 μ l
1.5 - - -	3.5 μ l

- | | | |
|----|--------------|----------------------------|
| 4. | LSN 9/6/94 | } T7 transcription + DNase |
| 5. | PI-5 9/6/94 | |
| 6. | PI-5 8/21/94 | |

1.5 - - -	3.5 μ l
1.5 - - -	3.5 μ l
1.5 - - -	3.5 μ l

7. λ Bst E II

9/7/1994

[Handwritten signature]

$m/M_m/M$ (amb. sy)

P1-5

P1-5 variant

water

8

9

Water



10x But

4

4

10x but

4

6x NTP/cap

20

20

2x NTP/cap

20

GTP 30 mM

2 (67% capped)

10

CE 80mm 1.5
NAE 80mm 2.5

1.5

LSN

0.5

DI-5

1

[Faint handwritten notes at bottom of page]

4

70x 2m

4

4

34-07P

3H-атр

1

1

40M

40 ml 6.26%

140

60

RNA gel

01-5/10+1
give pay
please, ←
precept, take

[illegible]

34

Electroporation RNAs

19.00

① 10 μ l of PI-5 8/21/94 + 10 μ l of LSN 9/7/94

②

settings: as in protocol,
with exception: 1.75 kV
10' at 37°C p.e.

+ purified
(20 μ l + 5 μ l NH₄Ac,
75 μ l cold EtOH,
15' -70°C
15' spin down,
wash w/ 75% EtOH,
dry, dissolved in 20 μ l

9/8/1994
Thursday

IFA: a-Lassa 45% positive
a-VEE 47% positive
supernatant

8:00

12:30 1 ml of ① } supernatant $\rightarrow$ -70°C
②

17:00 2 ml of ① supernatant $\rightarrow$ -70°C

Depletion of PI-5/NotI



BHK cells are seeded (~50,000/well - chamber of Pich 6
12 ml from roller bottle (5 ml of Trypsin + 30 ml EMEM
per 750 flask ~1.5 $\cdot$ 10⁶/ml cells

TRANSFECTION

PI-5 RNA 8/21/44 + 10 μ of Lsn RNA 9/7/44
 at RT, 1.75 kV, 10' at 37°C p.e.

PI-5 RNA 8/21/44 + 10 μ of Lsn RNA 9/7/44
 RNAs, but precipitated before electroporation
 (to remove NTPs)

purification by precipitation w/ ethanol
 and NH₄Ac drastically decreased
 transfection efficiency

EA	1 38/94 (40%) 20/37 (54%) 10/25 (40%) 11/22 (50%) 22/47 (47%)	2 2/40 (5%) 5/40 (12%)		
	1 9/24 (38%) 11/41 (27%) 17/30 (57%)	2 1/20 (5%) 1/23 (4%)		

mean (45%) (7%)

VE	1 15/38 (39%) 24/38 (63%) 14/39 (36%)	2 1/20 (5%) 2/30 (7%)		
	1 20/29 (69%) 11/27 (41%) 18/30 (60%)	2 3/40 (8%) 2/20 (10%)		

Mean (47%) (8%)

		18 hrs	36 hrs	60 hrs
		10 ⁻² dilution	10 ⁻² dilution	10 ⁻² dilution
Antibody	—	33	123	125
Conjugate	—	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴
		not detected	1	not detected

PV 3000 : EcoRI Apa I (React 4)

DNA	100 μ l	(~10 μ g)
10x Buf ④	50 μ l	
Water	340 μ l	
EcoRI	5 μ l	(60u)
Apa I	5 μ l	

⑥ hrs, + 50 μ l React ③
 + 450 μ l water
 + 5 μ l EcoRI
 overnight

fragments, smudge
 5 & 8 kb

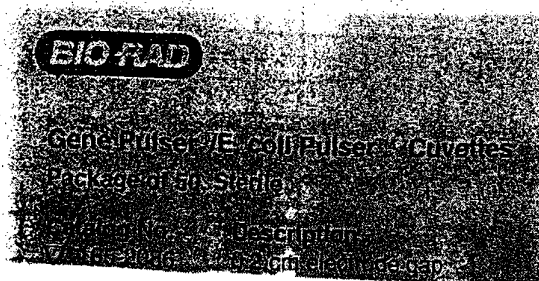
9/10/94
 Saturday

1000 — last supernatant from ① (Electroporation)
 ~80 hrs p.e.

9/11/94
 Sunday

Electroporation

10 μ l LSN 9/7/94 + 10 μ l LSN variant 9/9/94 + 20 μ l PI-5 8/21/9
 9.65 kV ; 10" RT p.e.
 1.2×10^7



9/12/94

9/12/94
Monday

ZPA cells electroporated 9/11/94

a - lassa (~ 10%)
a - VEE (~ 100%)after infection - $5.22 \cdot 10^8$ particles/ml
practically, no VEE antibodies
(negative test)(13502)
8133
536956°C pV3000 1) Bst E II
2) Sph I

Bst E II:	10 x (2)	25
pV3000 DNA		50
H ₂ O		175
Enzyme		5 (8 u/ml)
		56°C

Sph I	10 x (3)	25
0.1% BSA		25
H ₂ O		150
pV3000 DNA		50
Enzyme		3 (8 u/ml)
		37°C

pV3000

Bst E II

Sph I

1615 ÷ 1830

38

9/13/94
Tuesday

Transcriptions

LSN - mMmM (Amber)

P1-5 (Promega)

water 9
 10x buf 4
 2x ATP/cap 20
 GTP 30mM 2
 DNA/NotI 0.7
 U3 ATP 1
 10x Buz 4

40mM

4 hrs (!) - wrong?!

water 17
 5x buf 20
 100mM ATP 7.5
 100mM UTP 7.5
 100mM CTP 7.5
 100mM GTP 4.5
 25mM cap (Pharm) 24
 DNA/NotI 4
 U3 ATP 10
 10x Buz 10

100mM

m⁷G(5')ppp(5')G

27-4635-02

25 u

Lot 4054635021

For in vitro use only
 Store at -20°C

Made in USA

Pharmacia
Biotech

6%

54%

Nothing!

AP1 + AP2 } P1-5
 AP1 + AP2 }
 AP1 + AP2 }



no cleavage

Electroporation

16 μ l PI-5 8/21
16 μ l LSN 9/9 variant

1.7 kV, 10' RT

~~80-90% LSN~~
80-90% LSN
50-60% PI-5

9/14/94
Wednesday

permatant

probes

9/14 8:30 am
9/14 10:30 am
9/14 12:30
9/15 8:00
9/15 15:00
9/16 5:00 transcription

74 hrs p.e.
18 hrs p.e.
22 hrs
30 hrs

✓ 14 hrs p.e.
✓ 18
✓ 22
✓ 26
✓ 30
37.5
44.5
6 hrs p.e.
PI-5

LSN

water
5 $\times$ aut
25 mM NTP/-G
25 mM cap
8NA/NotI
43 ATP
10 $\times$ Bug
G 100 mM

8.9 μ l
8 μ l
12 μ l
4.8 μ l
0.7
1
4
0.5 μ l
10 μ l

water 1.4
5 $\times$ but 8
25 mM NTP/-G 12
25 mM cap 9
8NA/NotI 4
43 ATP 1
10 $\times$ Bug 4
G 100 mM 0.5
40 μ l

5 μ g / 20 μ
8 μ g / 10 μ

LSN

PI-5

LSN washed

PI-5 washed

OK
2.5%
2.1%

9/14/99
Wed

constant

probes

30 am
30 am
30
30
30

14 hrs
~~18~~ hrs
22 hrs
30 hrs

p.e.
p.e.

✓ 14 hrs p.e.
✓ 18
✓ 22
✓ 26
✓ ~~28~~ 30
37.5
44.5
62 hrs p.e.

PI-5

transcription

LSN

water
5x but
25mM NTP/-G
25mM cap
8NA/NOT I
43 ATP
10x 8u
G 100mM

8.9 µl
8 µl
12 µl
4.8 µl
0.7
1
4
0.5 µl
40 µl

water
5x but
25mM NTP/-G
25mM cap
8NA/NOT
43 ATP
10x 8u
G 100mM

14
8
12
9
4
1
4
0.5
40 µl

5/19/20
8/19/20

LSN

PI-5

LSN washed

PI-5 washed

OK
2.5%

2.1%

EcoRI
PV3000

25x EcoRI can't!

PV3000

Transcription

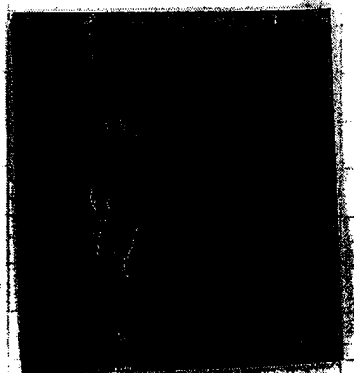
	<u>pV3000</u>
water	8.9
5x buf	2
25mM NTP/G	12
25mM cap	4.8
DNA/Not I	0.7
³ H ATP	1
10x BnZ	4
100mM GTP	0.6
	40 μ l

7/15/94

20569.04

0.95%

242.3578



RNA
pV3000 pV3000 M-RNA ladder BRe
+DNase



pV3000 kpn I
7 μ l on gel

(P2)

14 μ l water2 μ l pV3000/kp2 μ l Lig buf + 12 μ l T4 DNA

o/n 4°C

(10 μ l / 50 μ l of c
B

T. ...

pV3000

OK

p1-5

OK

7.9
8
12
4.8
1.7
1
4
0.6

(5.8)
8
12
(5.4)
(2)
1
4
(0.8)

6
8
12
7.2
1.2
1
4
0.6

(3.9)
8
12
(8.4)
(2)
1
4
(0.7)

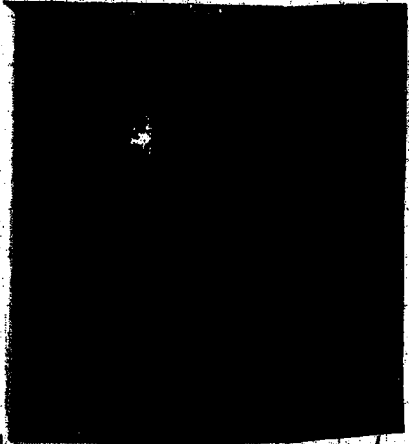
40 ml

40 ml

18688.0

0.66%
0.62%

1.37%



T4 polynucleotide treatment

Apa I / ~~Spa I~~

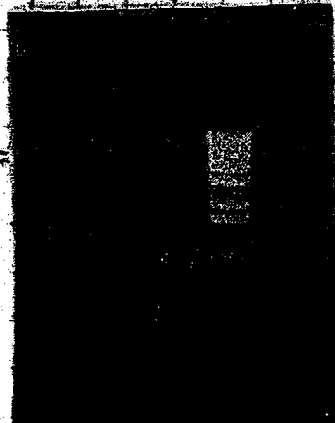
pV3000

Apa I / ~~Spa I~~

Water
RNA / ~~Spa I~~
10x B4
100 mM dMTP
T4 (P)

40
3
5
0.5
1

50

E. coli
ATP

20
25
5
0.5
1

50

RT 20' + 150 μ l water
+ 200 μ l phenol-chl
+ tRNA
+ NaCl

1.2
 4.8
 1.7
 1
 4
 0.6

(5.4)
 (2)
 (0.8)

7.2
 1.2
 1
 4
 0.6
 40 ml

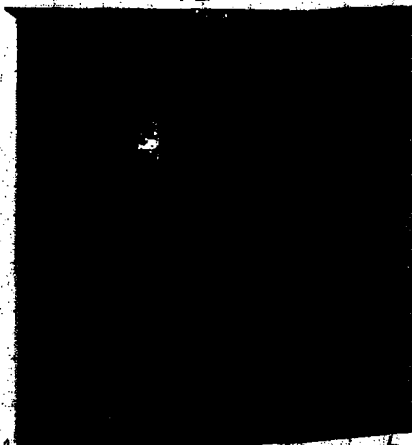
(8.4)
 (2)
 (0.7)

688.0

151

0.62%
 0.62%

1.37%



T4 polymerase treatment

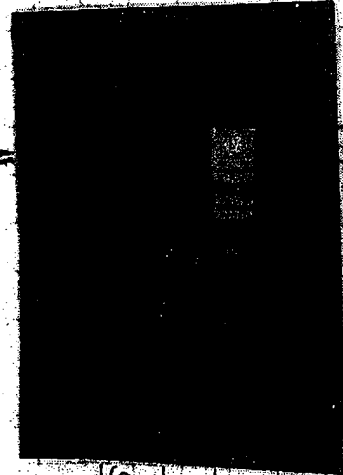
Ap I / ~~Sp I~~

pv3000

Ap I / ~~Sp I~~

water
 DNA / ~~Sp I~~
 10 x B4
 100 mM dAMP
 T4 (P)

40
 3
 5
 0.5
 1
 50



20
 25
 5
 0.5
 1
 50

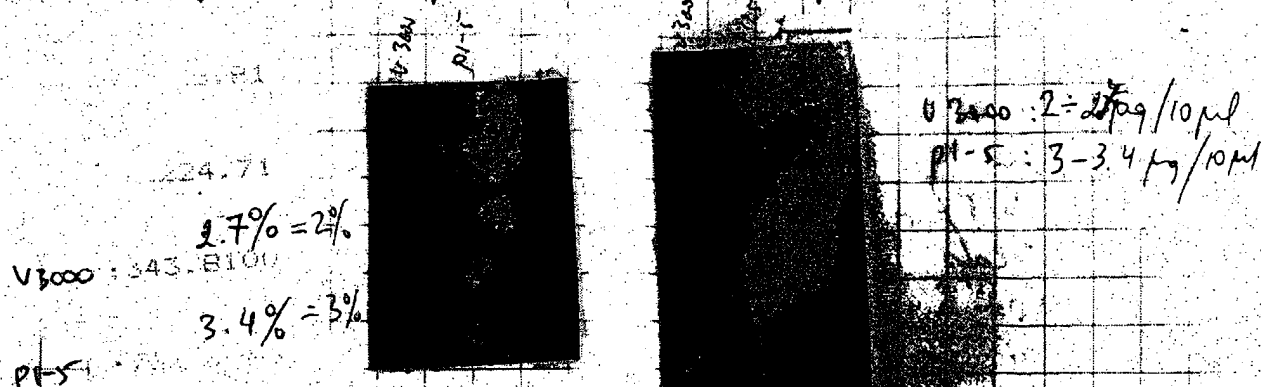
RT 20' + 150 µl water
 + 200 µl phenol-cl
 + tRNA
 + NaCl
 + EtOH 500 µl 20'

1.2
 4.8
 1.7
 1
 4
 0.6



9/16/94
Friday

1) checking yesterday's transcription



2) cleavage Kpn I + ④ React + 0.01% BSA

④ 10 μl on the gel

Ligation:

30 min
37°C

W' at 65° → 70°

2 μl of ④

15 μl of water

2 μl ~~ATP~~ Lipase buf

1 μl Lipase (T₄)

③ from P2: (K)/100 ∞
(P2)/100 ∞
(P2)/100 many

Kpn I: phosphorylated; 65°C 15';
genotyping; cur. 2)

4) IFA for α-lassa (of BHK infected w/replicon)
IFA: 14 hrs post infection (LSN + P1-5)
α-lassa # 288 conjugate LSN + P1-5 helper

lassa (infection) IFA 9/16	0.8 · 10 ⁵ /ml 14 hrs p.e.	1.1 · 10 ⁵ /ml 18 hrs p.e.	1.5 · 10 ⁵ /ml 22 hrs p.e.	3.1 · 10 ⁵ /ml 26 hrs p.e.
14 hrs post infection	2.6 · 10 ⁵ /ml 36 hrs p.e.	1.4 · 10 ⁵ /ml 37 hrs p.e.	0.4 · 10 ⁵ /ml 44 hrs p.e.	not detected 110 negative control not infected BHK cells

assayed
positive
negative
BHK cells
with

Trans
off
est
on

V3000

PI-5

2.7% = 2%

3.4% = 3%

V3000 : 2 = 2.7 pg/10 µl
PI-5 : 3-3.4 pg/10 µl

A 058

2. cleavage Kpn I + ④ react + 0.01% BSA

⑩ µl on the gel

Ligation: 2 µl of ⑥
15 µl of water
30 min
37°C
1 µl ~~lipase~~ Lipase buf
1 µl Lipase (T4)

from P2: ⑥/1000 ∞
P2/900 ∞
P2/100 many

Kpn I: purchased; 65°C 15';
genotyping; cur. 2)

4) IFA for a-lassa (of BKK infected w/repicarb particles)
IFA: ⑭ hrs post infection LSN + PI-5
a-lassa # 288 conjugate LSN + PI-5 helper

lassa (infection)	0.8 · 10 ⁵ /ml	1.1 · 10 ⁵ /ml	1.5 · 10 ⁵ /ml	3.1 · 10 ⁵ /ml
IFA	14 hrs p.e.	18 hrs p.e.	22 hrs p.e.	26 hrs p.e.
9/16				
14 hrs post infection	2.6 · 10 ⁵ /ml	1.4 · 10 ⁵ /ml	0.4 · 10 ⁵ /ml	not detected
	30 hrs p.e.	37 hrs p.e.	44 hrs p.e.	110" negative control, not infected BKK cells

amount of particles/ml of supernatant of BKK cells trans with LSN repl PI-5 heli
Transfection efficiency estimation as 80-90% 50%

9/13/64

BKK cells were subconfluent before infection

Compare to 9

5.22 · 10⁵/ml
when efficiency was 100% for both

IFA. (20 hrs) p.i.

LSN replicon
PI-5 helper

LSN 20 hrs 9/16	$3 \cdot 10^8$ /ml 14 hrs p.e.	$1 \cdot 10^5$ /ml 18 h	$1.4 \cdot 10^5$ /ml 22 hrs p.e.	$3.6 \cdot 10^5$ /ml 26 hrs p.e.
	$1 \cdot 10^5$ /ml 0 hrs p.e.	$2.8 \cdot 10^5$ /ml 37 hrs p.e.	$6.4 \cdot 10^5$ /ml 44 hrs p.e.	HC "0"

$6.5 \cdot 10^5$ /ml 68 hrs p.e.

see 9/20 IFA

Precipitation and cleavage w/ Arr 2 of

P3 and P4

~~Δp3/ΔstH~~~~Δp4/ΔpIT~~

20 μl + 80 μl water
 + 100 μl phenol:chloroform
 sup + ~~10~~ μl NaCl 1 M
 + 1 μl 10 μg/ml tRNA
 + 300 μl EtOH

-70°C 20' spin, wash ~~10~~ 2x 75% EtOH; dry, dissolve
 in 10 μl water + 2 μl Reagent 2 + 1 μl Arr 2

transformation into DMS (max efficiency)

Do not forget to check the same supernatants
 (14; 18; 22; 26; 30; 37; and 44 hrs p.e.)
 undiluted, for VEE protein!

LSN PI-5
replicon helper

9/18/94
Sunday

T4 polymerase

Apex	15 t
DNA	45 μ l
10x T4 buf	5 μ l
100 mM dNTP	0.5 μ l
T4 (P)	1
	50

Apex	8 μ l
	10 μ l
	5
	0.5
	1
water	35
	50

20' RT

+150 μ l water + 200 μ l phenol-chloroform,
 1' vortex 5' spinning;
 + 30 μ l iM NaCl + 2 μ l tRNA 10 mg/ml
 + 600 μ l EtOH 30' - 70°C
 dissolve in 10 μ l H₂O

4 μ l $\rightarrow$ gel

Electroporation

1 (CSN) 10 μ l 9/4 variant 5 μ g/10 μ l
 2 10 μ l 9/4 2-2.5 μ g/10 μ l
 3 10 μ l 9/4 1-1.5 μ g/10 μ l
 4 10 μ l 9/4 0.5-0.75 μ g/10 μ l

30 μ l 9/15 2-2.7 μ g/10 μ l

1.7 kV 10' at 10 p.e.

18-30

+ 10 μ l water
 + 2 μ l Lipene buffer
 + 2 μ l T4 DNA ligase

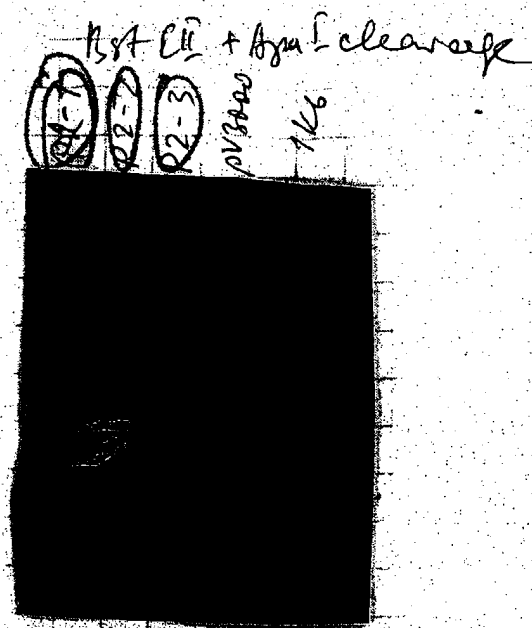
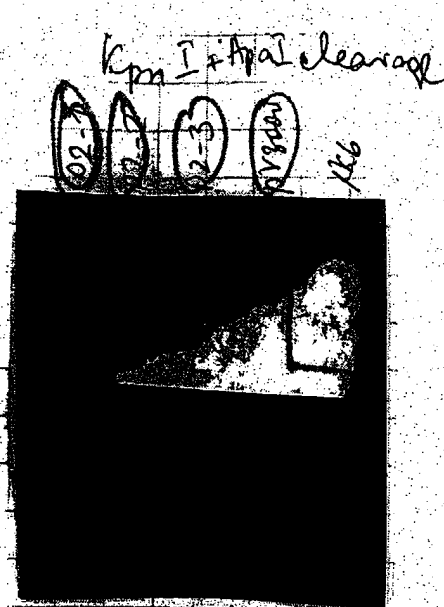
LSN + pV3000 as a helper

8 hr	p.e.	-	14 hrs p.e.
12 hr		-	~~18~~ hrs p.e.
16 hr		-	22 hrs p.e.
20 hr		-	26 hrs p.e.
24 hr		-	30 hrs p.e.
8 hr		-	38 hrs p.e.
15 hr		-	45 hrs p.e.
α-lassa		(~ 60-80% ; up to 90%)	
α-VEE		(~ 50-70% ; maybe 60-70%)	
α-lassa		(~ 60-80% ; 90%?)	
α-VEE		(60-70%)	

P2 miniprep (V3000 Δ KpnI) No. 1-16
STET-LSVET method

cleaved KpnI
 dissolved in 50 μl of water
 2 μl of DNA + 50 μl lye. Mix { (1:10 10x Reagent ④)
 1:10 0.1% BSA }

9/20/1994
Tuesday



(14) & (15) in "replicon" box at -70°C freezer
these are frozen E. coli cultures
of $\Delta\text{Bst EII} - \text{Apa I}$ } of mutant "replicon"
 $\Delta\text{Spl I} - \text{Apa I}$ } helper

Bacterium 2 ml - LB cultures of:

1. P2-1
2. 14 } makes Spl I
3. 15 } makes Bst EII

P2-1

$\Delta\text{Kpn I}$ pV3000 helper

8:30-10:30 Infection with vaccinia particles and
10:30 IFA LSN + pV3000 (particles)

(14) & (15) in "replicon" box at -70°C freezer
 there are frozen E. coli cultures
 of $\Delta\text{BstEII} - \text{SmaI}$
 $\Delta\text{SptI} - \text{SmaI}$ } of "mutant" ~~replicon~~ ^{helper} ~~genon~~

Saccarose 2ml - LB cultures of:

A 063

1. -P2-1
2. 14 } Mike's Spt I
3. 15 } Bst EII

P2-1

ΔKpnI pV3000 helper

8:30-10:30 Infection with recombinant particles and

18:30 IFA LSN + pV3000 (particles)

"8 hrs p.i."

8-10 hrs
p.i.

10^{-3} dilution of supernatant

LSN + PV3000 RNAs transplanted, temps collected

6/20	○	○	○	1(+) K272K 0.1-10 ⁷ /ml
14 hrs p.e.	14 hrs p.e.	18 hrs p.e.	22 hrs p.e.	26 hrs p.e.
60 hrs post infection	0.3-10 ⁵ /ml 30 hrs p.e.	○ 38 hrs p.e.	0.5-10 ⁵ /l 68 hr. see 91	○ "NC" "Bike" "L. medium"

10⁻³ dilutions
of supernatant
100 µl of diluted
sup,
2 hrs infection
at +37°C
+ 300 µl medium
incubation
continued at
37°C

α-Lassa #288 conj.

LSN+PI-5

same,
VEA was done
8 or 24 hrs post infection

LSN + PV3000 RNAs

	○	nd	nd	○
24 hrs post infection	3-10 ⁴ /ml	nd	6-10 ⁴ /ml	nt "nc"

α-Lassa #288 conj.

LSN+PI-5

9/21/94
Wednesday

	nd	nd	nd	nd
8 hrs p.i.	2-10 ⁵ /ml	nd	nd	nd

α-VEE

α-mouse conj.

~~not tested~~
~~not tested~~
~~not tested~~

TRANSCRIPTIONS

LSA

V8000

water	8.8
5x But	8
25mm BP/-G	12
25mm cap	4.8
20x BTI	0.7
4x BP	1
6 BP 100mm	0.7
10x Eng	4

cap/G 1.7:1

~~5.8~~ 7.7
8
12
~~6.3~~ 4.8
2
1
0.8
4

1.5:1

LEGATIONS

P3 Bst (V8000 & Bst II - Apal)

P4 Spl (V8000 & Spl I - A)

water	10
ANA/TuP	6
10x Lipase	2
4x ANA Ligs	2
	20 pl

10	
6	
2	
2	
	20 pl

9/22/94
Thursday

i)

PM 12:58

5% Cassa

6% V3000

Transcriptions

LSN can be used
(~0.5 µg/µl ⇒ ~15 µl per electroporation)

V3000 - bad quality RNA,
pV3000/Not I is (twice) phenol
treated and precipitated

2)

Transformation

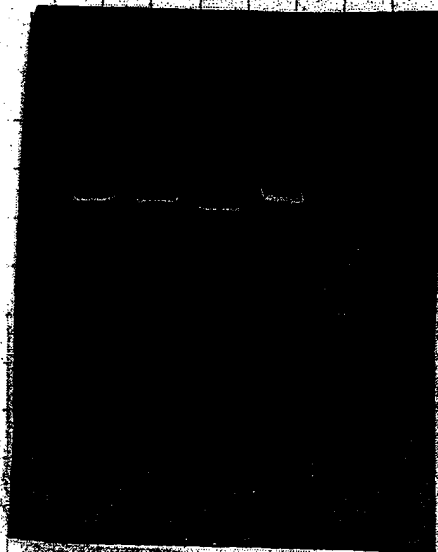
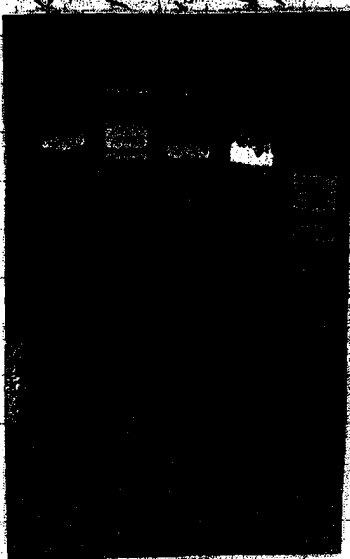
P3 fRST E II Apr L/pV3000
P4 (S Sple L Apr I/pV3000)

3)

DNA isolation

P2-1

14 } Mike's helper
15 }



degradation
of DNA

4) also see Not I analytical

50

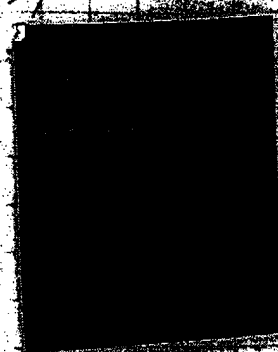
7/23/94

P4
 Δ Spl I Apr / V3000
 T4 (P)
 T4 (L)

(2) clones

Preparative cleavage Not I

1) P2-1
 2) 14



V3000 / 0 Apr I Spl I / T4 and / purified w/ Wizard
 V3000 / Δ Apr I Spl I / T4 PCR prep with

LIGATION

	P3/Bst	P4/Spl
water	25	25
SA A	4.5	20
10 x L	5	5
Ligase	1.5	1.5
	50 pl	50 pl

30' at 37°C

50 pl → E. coli DH5α transformation

P3/Bst : 33 clones / per 200 pl / plate
 P4/Spl : none

9/24/94
Saturday

TRANSCRIPTIONS OK

	LSA	V3000	RSK	PI-5
W000	8.8	8.8	3.9	3.9
W001	8	8	8	8
W002 NTP/-G	12	12	12	12
W003 M cap	4.8	4.8	8.4	8.4
W004 A/NO+I	0.7	0.7	2	2
W005 ATP	0.9	0.9	0.9	0.9
W006 100 mM	0.7	0.8	0.7	0.7
W007 10x Eng	4	4	4	4

LSN { 3.8%

V3000 { 2.9%

RSK { 4.25%

PI-5 { 3.1%



9/25/94
Sunday

ELECTROPORATION 20:00

- | | | | |
|----|-------------|-----------------------------------|------------------------------------|
| 1. | V3000 | 16 μ l 9/24 + 10 μ l 9/14 | Lassa NO |
| 2. | LSN + V3000 | — / — | + 10 μ l 9/24 + 7 μ l 9/24 |
| 3. | LSN + RSR | 10 + 7 Lassa | RNA RSR 15 μ l 9/24 |
| 4. | LSN + PI-5 | 10 + 7 Lassa | RNA PI-5 20 μ l 9/24 |

EE (5.54 μ l)	①	V3000	2.9%	16 μ l 9/24 + 10 μ l 9/14	0.9%
EE (5.54 μ l)	②	LSN + V3000	16 μ l (2.9%) 9/24 + 10 μ l (0.9%) 9/14	10 μ l (3.8%) 9/24 +	
SA (6.38 μ l)	③	LSN + RSR	15 μ l (4.25%) 9/24		
1-5 (6.2 μ l)	④	LSN + RSR	20 μ l (3.1%) 9/24		

9/26/94

8:00 (12 hr p.e.) 0.5 ml sup. samples taken

12:00 (16 hr p.e.)

16:00 (20 hr p.e.)

19:20 (24 hr p.e.)

24:00 (30 hr p.e.)

9/27 8:00 (38 hr p.e.)

9/27 14:30 (46 hr p.e.)

9/28 19:20 (~ 51 hr p.e.)

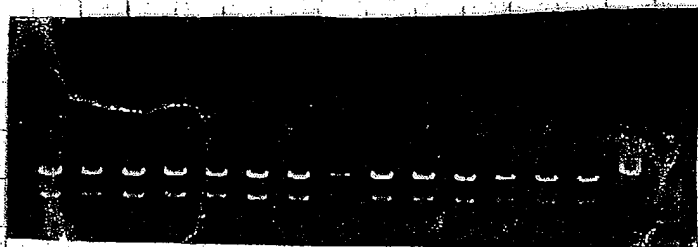
9/28 8:30 (~ 69 hr p.e.)

9/29 8:20 (~ 88 hr p.e.)

9/30 8:20 Mini Prep P4/1 ÷ 3
(~ 112 hr p.e.) P3/1 ÷ 21

cleavage

all
mini prep / gel
deletion
helper
(1)(2)(3)



P3 1 ÷ 14

V3000 1 kb



P3 1 ÷ 14

1FA a-lasse

① "0" ② 50÷70%

50÷70% 50÷70%

③ ④

a-VEE

① 40÷50% ② 40÷50%

③ 40÷50% ④ 40÷50%

TRANSCRIPTIONS

① LSN

② pV3000 as in 9/24



LSN

V3000

LSN 4.4%V3000 2.2%P4-1^{OK}

a-lasse & Ana? - Sme? below

54

9/27/94
Tuesday

TRANSCRIPTIONS

OK

	LSN	V
water	8.8	8
5x buf	8	8
25 mM NTP/-6	12	12
25 mM cap	4.8	4.8
DNA/Not I	0.7	0.7
H ³ UTP (1:1 ethanol/water)	1	1
100 mM GTP	0.7	0.8
10x E. coli mix	4	4

at 37°C

9/28/94
Wednesday

578%

4.88%

OK

P4-1 helper (Δ Spe I - Spe I) isolation

pv 8000

⑧ ml of 1:10

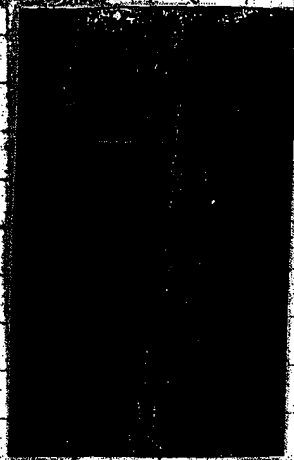
NotI SpeI



Uncleaved



NotI



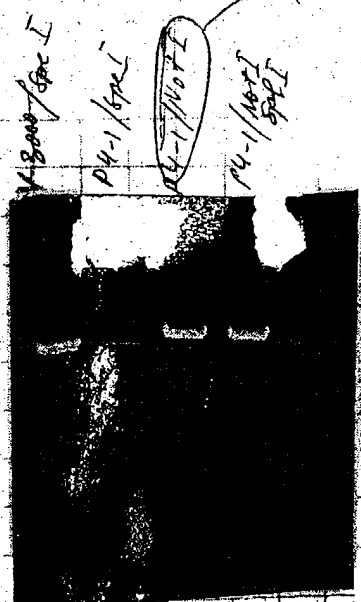
NotI SpeI
uncleaved
(17°C?)
SpeI does
not exist!!!
Δ SpeI Apr I!!!

9/29/94
Thursday

P4-1 NotI precipitation
dissolved in 50 μl water
1 μl - 1% gel

for transcription

9/30/94
Friday



pv 8000 for I

P4-1/NotI

P4-1/NotI

P4-1/NotI

P4-1/NotI

TRANSCRIPTIONS

	P2-1	P4-1	14	15	CSN
water	9.1	8.3	8.3	8.3	8.8
SX Buf	8	8	8	8	8
25 mM NTP/G	12	12	12	12	12
25 mM Mg	4.8	5.6	5.6	5.6	4.8
RNA Pol I	1	1	1	1	0.7
H ₃ UTP	1	1	1	1	1
100 mM GDP	0.7	0.7	0.7	0.7	0.7
10x RNase	4	4	4	4	4
	1:1.7	1:2	1:2	1:2	1:1.7

P2-1
5.73%

P4-1
7.50%

14
5.86%

15
0.89%

CSN
1.33%

A1404
A3551
A5023
RNA Pol I
RNA Pol II
RNA Pol III
CSN/Rep1.2

P2-1
P4-1
14
15
CSN

A SPEC
APOL

WAKE DNA...

10/1/1994

10/2/94

BHK cells are ~ 70% confluent

ELECTROPORATION

 $2 \cdot 10^7$ cells/ml, 1.50 kV
 $\sim 10,000,000$ cells/plate
① NC (NO RNA)

12.00

I

② LSN + P2-1 (5 kpm I)

LSN: 1.7 μ l of (3.8%) $\Rightarrow$ 0.646 μ g
 10 μ l of (2.5%) $\Rightarrow$ 2.5 μ g
 6 μ l of (5.78%) $\Rightarrow$ 3.468 μ g
 $\star$ 6.614 μ g

~~total~~P2-1: 12 μ l of (5.73%) $\Rightarrow$ 6.876 μ g~~total~~③ P2-1 12 μ l of (5.73%) $\Rightarrow$ 6.876 μ g~~total~~

④ LSN 1.7 μ l (3.8%) $\Rightarrow$ 0.646 μ g
 10 μ l (2.5%) $\Rightarrow$ 2.5 μ g
 6 μ l (5.78%) $\Rightarrow$ 3.468 μ g
 $\star$ 6.614 μ g

~~total~~⑤ LSN + V3000

LSN: 1.7 μ l of (3.8%) $\Rightarrow$ 0.646 μ g
 6 μ l of (2.5%) $\Rightarrow$ 1.5 μ g
 8 μ l of (5.78%) $\Rightarrow$ 4.624 μ g

 $\star$ 6.77 μ g~~total~~

V3000: 2.5 μ l (2.7%) $\Rightarrow$ 0.675 μ g
 2 μ l (2.9%) $\Rightarrow$ 0.58 μ g
 10 μ l (4.88%) $\Rightarrow$ 4.88 μ g

 $\star$ 6.135 μ g $\star$ 1.1

II

LSN

$$\begin{aligned}
 1.7 \mu\text{l of } (3.8\%) &\Rightarrow 0.646 \mu\text{g} \\
 10 \mu\text{l of } (2.5\%) &\Rightarrow 2.5 \mu\text{g} \\
 6 \mu\text{l of } (5.78\%) &\Rightarrow 3.468 \mu\text{g} \\
 &\quad + 6.614 \mu\text{g} \\
 &\quad \text{---} \\
 &\quad \text{total}
 \end{aligned}$$

P2-1: $12 \mu\text{l of } (5.73\%) \Rightarrow 6.876 \mu\text{g}$

P2-1 $12 \mu\text{l of } (5.73\%) \Rightarrow 6.876 \mu\text{g}$

LSN

$$\begin{aligned}
 1.7 \mu\text{l } (3.8\%) &\Rightarrow 0.646 \mu\text{g} \\
 10 \mu\text{l } (2.5\%) &\Rightarrow 2.5 \mu\text{g} \\
 6 \mu\text{l } (5.78\%) &\Rightarrow 3.468 \mu\text{g} \\
 &\quad + 6.614 \mu\text{g} \\
 &\quad \text{---} \\
 &\quad \text{total}
 \end{aligned}$$

(5) LSN + V3000

LSN

$$\begin{aligned}
 1.7 \mu\text{l of } (3.8\%) &\Rightarrow 0.646 \mu\text{g} \\
 6 \mu\text{l of } (2.5\%) &\Rightarrow 1.5 \mu\text{g} \\
 8 \mu\text{l of } (5.78\%) &\Rightarrow 4.624 \mu\text{g} \\
 &\quad + 6.77 \mu\text{g} \\
 &\quad \text{---} \\
 &\quad \text{total}
 \end{aligned}$$

V3000:

$$\begin{aligned}
 2.5 \mu\text{l } (2.7\%) &\Rightarrow 0.675 \mu\text{g} \\
 2 \mu\text{l } (2.9\%) &\Rightarrow 0.58 \mu\text{g} \\
 10 \mu\text{l } (4.88\%) &\Rightarrow 4.88 \mu\text{g} \\
 &\quad + 6.135 \mu\text{g} \\
 &\quad \text{---} \\
 &\quad \text{total}
 \end{aligned}$$

(6) V3000

$$\begin{aligned}
 2.5 \mu\text{l } (2.7\%) &\Rightarrow 0.675 \\
 2 \mu\text{l } (2.9\%) &\Rightarrow 0.58 \\
 10 \mu\text{l } (4.88\%) &\Rightarrow 4.88 / 6.135 \mu\text{g} + \\
 &\quad \text{---} \\
 &\quad \text{total}
 \end{aligned}$$

58

Supernatant Samples

16⁰⁰

20:20

9.150

10/3/1994 when IPA was done 6:10

00:

10

18h IPA a-L
COO & slide (1)(4)
LSN

for each ~ 30-40% conc

- larva replican
- Δ KpnE V8000 helper

a-VEE

P2-1 doesn't work

dilutions

VEE 1:25

Gm mouse 1:25

(1) NE 0

(2) high background
LSN P2-1 "0"

(3) 0

(4) low background

(5) low background

(6) 20-40%

20-40%

(7) 20-40%

(8) linear

Δ KpnE

Δ KpnE
split

p18000: 63055; 7200

p2-1: 12008

p4-1: 2754; 7200

14: 2754; 7200

15: 7679

p1-5: 6513

RSR: 6513

63055

12008

2754

2754

7200

7679

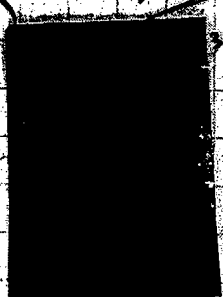
6513

6513

6513

cleared 2nd A templates
transcription

3000/lat II (partial)



30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

30

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30

30

30

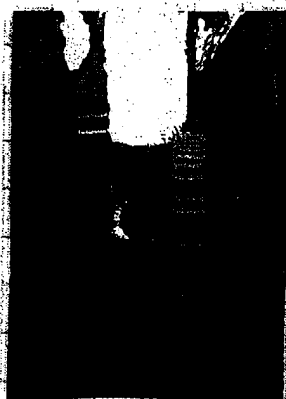
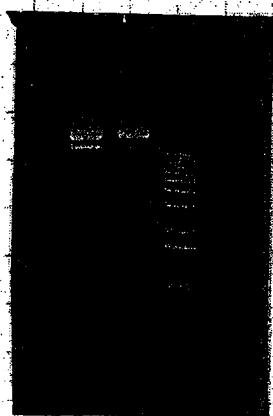
30

BVK - 38 pass.

IFAs of supernatants from electroporation
(see previous page, p. 60)

10/5/1994 Wednesday

pV3000 - Cat II in Beckinger - Mannheim (A) buffer



Att II
partial

Electroporation 14:00 as in p

10/6/1994

Samples

1) 7.728 μ g SN 5 μ l of 0.5 μ g/ μ l + 6 μ l
8.28 μ g P1-5 17 μ l of 0.34 μ g/ μ l + 6 μ l

4 μ l of 0.44 μ g/ μ l
1 μ l of 0.14 μ g/ μ l

2) 7.728 μ g SN as in 1)
8.0 μ g SR 20 μ l of 0.4 μ g/ μ l

3) 8.28 μ g P1-5 as in 1)

4) 6.58 μ g SN 5 μ l of 0.5 μ g/ μ l + 2.5 μ l of 0.578 μ g/ μ l + 6 μ l 0.44 μ g/ μ l
6.83 μ g 3000 14 μ l of 0.488 μ g/ μ l

A5

pv3000 cleavage with Act II

Water	160 µl
10x Rcast 2	20 µl
RV3000	10 µl
cat 4	3 µl

at 37°C	2.55
---------	------

8/9/1994

a-lassa

a-VEF
a-RABBIT

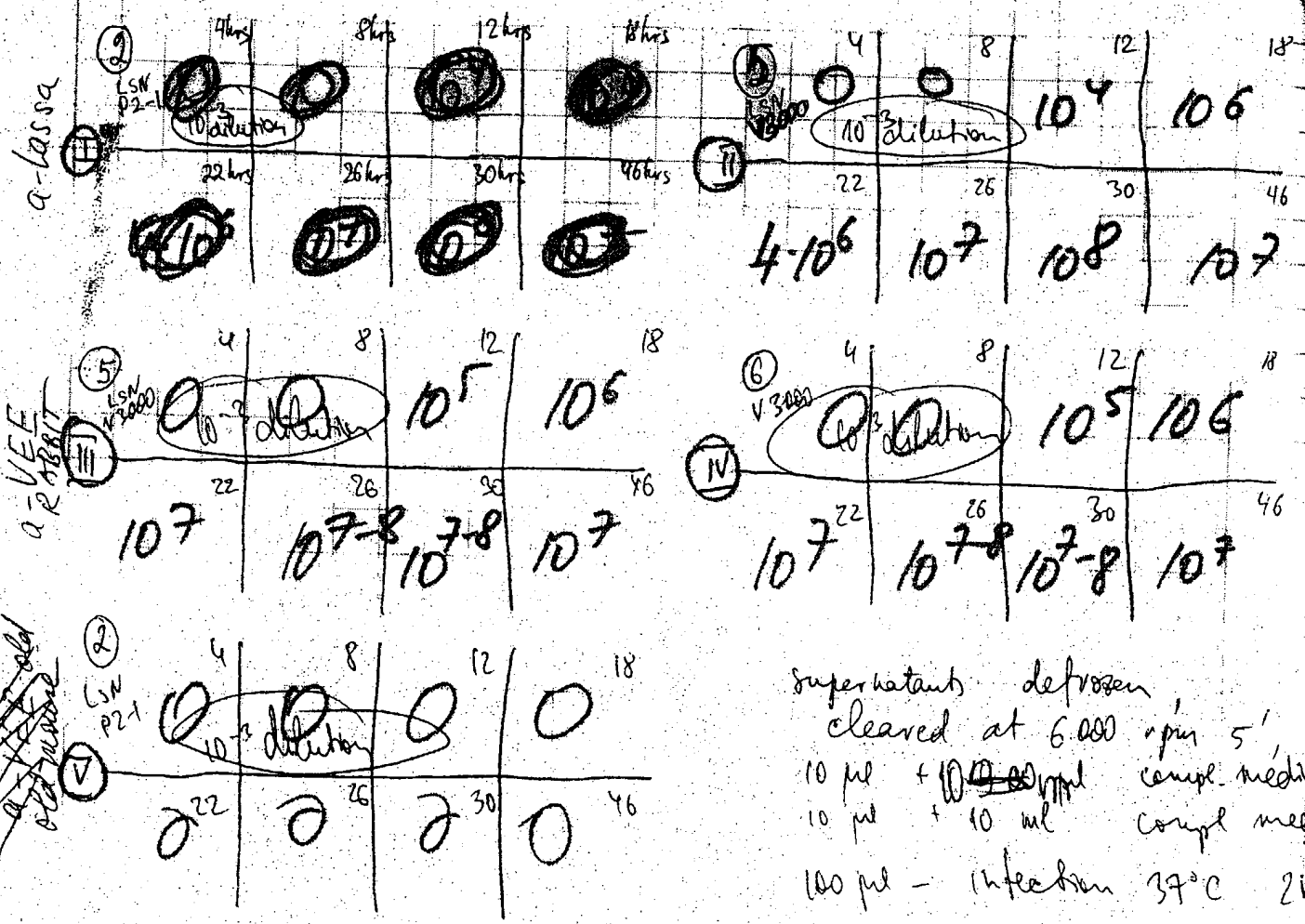
②
LSN
#21

supernatants defrozen
cleared at 6000 rpm 5'
10 µl + ~~100 µl~~ 100 µl compl m
10 µl + 10 µl compl n
100 µl in 10 µl compl

7/4/1994

A 080

No
cleavage
o/n (~20 hrs)!!!



supernatant defroze
cleared at 6,000 rpm 5'
10 μ l + 10 μ l compl. media
10 μ l + 10 μ l compl. media
100 μ l - infection 37°C 2h
+ 300 μ l compl. media 4h

Supernatant samples

1800

(11.5 hrs. p.e.)

2200

(11.5 hrs. p.e.)

130

(11.5 hrs. p.e.)

(air bubbles (transcript) preventing counting??)

- ① 10 µg RNA (SN+RHS) medium pink, many fewer cells than in ②
 ② 15.7 µg RNA (SN+RSL) medium yellow, many cells
 ③ 8.3 µg RNA P1-5 yellow
 ④ 13.4 µg RNA (SN+RHS) yellow

- ① ③ still pink (1000) - yellow but growth is attached to 8 tubes, the same in

medium is the same about the same, number of attached cells is less than in other

17/94

800

(18 hrs. p.e.)

1200

(22 hrs p.e.)

1600

(26.5 hrs)

1900

(29.5 hrs)

2400

(34 hrs)

930

(13.5 hrs)

1400

(17.5 hrs)

1400

(17.5 hrs)

medium is the same cells about 4 samples

slides:

2-VEE

①	②
20-30%	~30-40%
③	④
0%	~40%

18/94
19/94
11/94

930

(13.5 hrs)

1400

(17.5 hrs)

1400

(17.5 hrs)

presence of transcription factor when added (30% increase in VEE activity)

10/7/94 TRANSCRIPTIONS

	CSN	V3000	P2-1	13
water	18.6	8.8	8.3	7.7
5x buf	16	8	8	8
25mM NTP-G	24	12	12	12
25mM Cap	9.6	4.8	5	5.6
RNA/Not I	1.5	0.7	1	1
U3UTP	1	1	1	1
100mM GTP	1.4	0.8	0.7	0.7
10x E. coli mix	8	4	4	4

5-104

LSN undiluted 1:10 (NC)

~~28~~ 4	~~28~~ 8	~~28~~ 12	~~28~~ 18
220	26	30	36

A 082

VEE 10-2
30 hrs NC

4.6

80

many false-positive,
probably because of
some cross-reactivity
of Lassa with
VEE
3.5 slides

Lassa

Infection with supernatants from electroporation 10/2/94

a-Lassa

③ LSN + V3000; dil. 10^{-4} dilution of supernatant

12 hr/ 10^{-4}	18 hr/ 10^{-4}	22 hr/ 10^{-4}	26 hr/ 10^{-4}
wt	wt	wt	wt
30 hr/ 10^{-4}	34 hr/ 10^{-4}	44 hr/ 10^{-4}	80 hr/ 10^{-4}
$\sim 0.10^5$	$2 \cdot 10^5$	$3 \cdot 10^5$	$3 \cdot 10^5$ particles/ml

④ LSN; dil. 1:10 (10^{-1}); NC

4 hr/ 10^{-1}	8 hr	12 hr	18 hr
○	○	○	○
22 hr	26 hr	30 hr	34 hr
○	○	○	○

⑤ LSN + V3000; dilution 10^{-2}

4 hr/ 10^{-2}	8 hr	12 hr	18 hr
$\sim 1 \cdot 10^3$	$\sim 1 \cdot 10^3$	$2 \cdot 3 \cdot 10^3$	$7 \cdot 8 \cdot 10^3$
22 hr	26 hr	44 hr	80 hr
$3.5 \cdot 10^4$	$3.5 \cdot 10^4$	$2.2 \cdot 10^5$	$2 \cdot 10^5$

⑥ LSN (control)

44 hr	100 hr
○	○
80 hr	100 hr
○	○

many false-positive,
probably because of some
cross-reactivity with
VEE antisera
of a-Lassa-conjugate

:9234

:8796

1.46%

1.51%

:133.1153

:19299.45

:16868.83

1.57%

2.15%

:304.3564

:363.4319

:18174.55

0.4%

:74.44427

0.2%

① LSN + V3000

② ~~V3000~~

12 hr	18 hr	22 hr	26 hr
12 hr	18 hr	22 hr	26 hr
30	36	46	80
~300	~1000	~800	~100
$1 \cdot 10^5$	$2 \cdot 10^5$	$3 \cdot 10^5$	$3 \cdot 10^5$

supernatants from operation 10/2/94

⑤ LSN + V3000, dilution 10^{-2}

4 hr	8 hr	12 hr	18 hr
4 hr	8 hr	12 hr	18 hr
~1.10 ³	~1.10 ³	2.3.10 ³	7.8.10 ³
22 hr	26 hr	44 hr	80 hr
3.5.10 ⁴	3.5.10 ⁴	2.2.10 ⁵	2.1.10 ⁵

⑥ LSN (continued); dilution 10^{-2}

44 hr	V3000 (sample ⑥)
44 hr	V3000 (sample ⑥)
○	at 10^{-2} dilution
80 hr	30 hrs p.c.
○	empty

many false-positive,
probably because of some
cross-reactivity with
VEE antisera
of a-lassa-conjugate

91 - VEE

⑤ LSN + V5000; dil 10^{-4}

12hr	18hr	22	26
less	less	less	less
30	36	44	80 hr p.e.
$\sim 10^6 - 10^7$	$10^7 - 10^8$	$10^7 - 10^8$	$\sim 10^7$

⑥ V5000; dil 10^{-4}

12	18	22	26
less	less	less	less
30	36	44	80 hr p.e.
less	$10^7 - 10^8$	$10^7 - 10^8$	less

④ LSN; dil. 10^{-1} ; NC

12	18	22	26
practically			
30	36	46	80 hr p.e.
			nothing

⑤ LSN + V3000; dil 10^{-2}

4	8	12	18
22	main	44	80 hr p.e.

⑥ V3000; dil 10^{-2}

4	8	12	18
22	main	44	80 hr p.e.

← Difficult to count

Since VEE often makes adjustment

10/8/44
Saturday

⑤ ~ 4 V $\sim 11^{-2}$
⑥ ~ 2 V $\sim 11^{-2}$

12	18	22	26
less	less	less	less
30	36	46	80
main $10^7 \div 10^8$	main $\sim 10^7 - 10^8$		

4	8	12	18
22	26	46	80

4 slides

LSN $\sim 10^{-1}$

IX

12	18	22	26

up out of 17

1.

TRANSCRIPTIONS

	LSN	V3000	P1-5	P2-1
Water	8.8	8.8	3.9	5.4
5X But	8	8	8	8
25 mM ATP-G	12	12	12	12
25 mM cap	4.8	4.8	8.9	4.8
25 mM NaCl	0.7	0.7	2	4
43 UTP (1:1 ethanol/water)	1	1	0.1	1
100 mM GDP	0.7	0.8	0.7	0.8
10x Enzyme	4	4	4	4
	40	40	40	40

1/12/94
Wednesday

} aise
0.2%

} V3000
0.2%

} P1-5
0.3%

} P2-1
2%

TRANSCRIPTIONS

water
 5x Buf
 25 mM MP/G
 25 mM cap
 DVA/Not I
 43 CLP
 100 mM GSP
 10 x Enzymes

L S N

8.8
 8
 12
 4.8
 1
 0.7
 0.7
 4
 40

V3200

8.7
 8
 12
 4.8
 1
 0.7
 0.8
 4
 40

PI-5

4.7
 8
 12
 8.4
 1.5
 0.7
 0.7
 4
 40

4 hrs
 37°C



lassa
 0.4%

V3200
 4.2%

PI-5
 6.8%



1 µl of LSN/Not I

10/13/1994

TRANSCRIPTIONS

LSN

OK

Water 9.4
 5x Buf 8
 25 mM NTP/G 12
 25 mM cap 4.8
 200 mM NaCl 0.40.5
 100 mM GTP 0.7
 10x Euzymix 0.7
 4

6.2%

TRANSCRIPTION

0.4990

① LSN + V3000

② LSN + P1-5

③ LSN + P2-1

④ LSN + V3000

⑤ LSN + V3000

⑥ P1-5

4.4 1.32 15.6.2 0.9 4.222 6.8 3.2

5 10 - 6 5 12 5 - -

5 10 - 6 - - - 10 -

5 10 - 6 - - - - 23

3 3 10 6 5 12 5 - -

3 3 10 6 5 12 5 - -

- - - - - 10 -

10/14/1994

Supernatants are taken

10/13 11:30 pm (5 hrs p.e.) cells are attached many cells or all of them alive
 10/14 8:30 am (14 hrs p.e.) all cells are attached, it seems, that for many cells we cannot see any be high, some areas of clumped cells,
 14:30 pm (20 hrs p.e.)
 do: 30 pm (26 hrs p.e.)
 10/15 8:30 am (38 hrs p.e.) → all cells to "swim" ①-⑤
 collected supernatants

IFA for evaluation of transfection efficiency
 (14 hrs p.e.) 1 LSN and 13000
 in wells w/ samples ①, ② & ③ (LSN + Helpers)
 cells shrank, come off the slide → they are washed or not attached
 and were removed when slides were put into washing tray w/ PBS
 too many cells (?)

a-lasse

① ~80% ④⑤	② no cells ⑥	③ no cells /	/
~80% ④⑤	⑥ /	/	/

a-VEE

① no cells ④⑤	② no cells ⑥	③ no cells /	/
70-80% ④⑤	⑥ /	/	/

TRANSCRIPTIONS

	LSN	13000	P2-1	R5R	P1-
enter	18.8	8.7	5.4	3.9	4.1
5x buf	16	8	8	8	8
25 mM ATP/G	24	12	12	12	12
25 mM cap	9.6	4.8	4.8	8.4	8.4
DNA/Not I	1	1	4	2	1.5
H3 UTP	1.3	0.7	1	0.8	0.7

10/14 8:30 am (14 hrs p.e.)
 14:30 pm (20 hrs p.e.)
 do 1:30 pm (26 hrs p.e.)
 10/15 8:30 am (38 hrs p.e.)

all cells are attached, it seems, that
 for many cells concentration may be
 high, some areas of submerged cells
 begin to "swim" (1)-(5)
 all supernatants
 collected

A 090

IFA for evaluation of transfection efficiency
 (14 hrs p.e.) LSN and V3000
 in wells w/ samples 1, 2 & 3 (LSN + Helpers)
 cells shrank, some of the slide $\Rightarrow$ they
 are washed or not attached
 and were removed when
 slides were put into washing tray w/ PBS
too many cells (?)

a-lasse

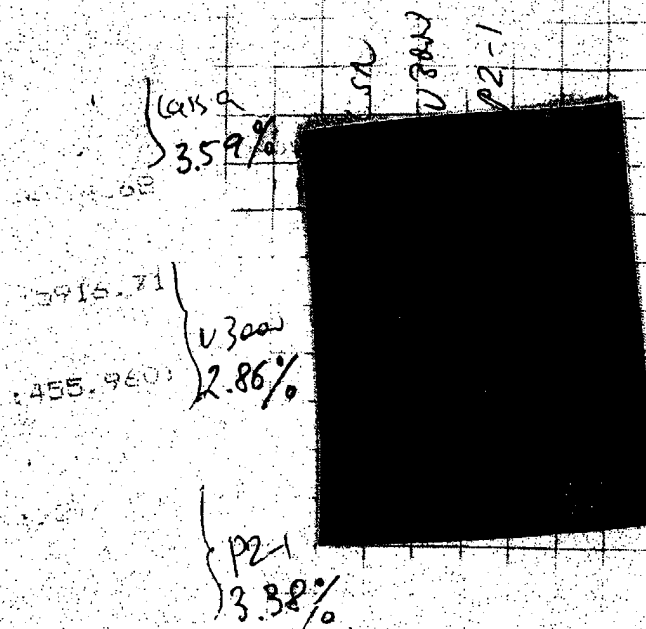
① ~80% ④⑤	② no cells ⑥	③ no cells /	/
④⑤	⑥	/	/

a-VEE

① no cells ④⑤	② no cells ⑥	③ no cells /	/
④⑤	⑥	/	/

TRANSCRIPTIONS

	LSN	V3000	P2-1	RSR	PI-
water	18.8	8.7	5.4	3.9	4.1
5x buf	16	8	8	8	8
25 mM ATP/G	24	12	12	12	12
25 mM cap	9.6	4.8	4.8	8.4	8.4
2NA/NotI	1	1	4	2	1.5
H3 UP	1.3	0.7	1	0.9	0.7
100 mM GDP	1.4	0.8	0.8	0.7	0.7
10 x Eus mix	8	4	4	4	4



9/15/1994

Infection w/ supernatant of BHK cells
 1 hr at 37°C infection in 100 μ l of solution
 (various dilutions), + 300 μ l of medium,
 incubate at 37°C

1700 medium added (300 μ l)

9/16/94

IPAs
 a-Gal

① USN + P2-1		5 hrs p.e.	14 hrs	26 hrs	
10 ⁻⁴ dilution		at	at	"0"	2.10 ⁵ (25%) i.u./ml
38 hrs p.e.		⑥ 5 hrs	④ 14 hrs	nc	
2.10 ⁵ (25%)		cells	10 ⁻² cells	cells, no supernatant,	
		"0"	(many)	"0"	1 hr medium
② USN + P1-5		5 hrs	14 hrs	20 hrs	26 hrs
10 ⁻⁴ dil.		0	0	1.10 ⁵ (25%)	6.10 ⁵ (25%) i.u./ml
38 hrs		nc	38 hrs	nc	
6.10 ⁵ (25%)		"0"	10 ⁻²	"0"	
			(many)	"0"	

③ USN + P2-1		10 dil.	14 hrs	20 hrs	26 hrs
all negative		5 hr			
		"0"	0	0	hr 0
		38 hr			
		0	nc	38	5 hr
			0	10 ⁻² cells	10 ⁻² dil.
				0	0

10/17/1999

IFA a-Casie
13 hrs pe.

① ~80% ② ~80%

③ ~80%

a-VEE

① ~80%

② ~1%

③ ~80%

- why IT? no more
... (15 test p.c.)

denaturing RNA gel

1) P1-5

1 μ l

2) P4-1

1 μ l

3) RNA ladder

1 μ linstead
V3000

I used 272.3 - triple mutant

10/18/99

Infection 22:00

① - 4 10-4 5 wt	14 wt ⑥ - 5 Kouly ⑦ - 10⁵ / ml i.c.u. / ml (+ 25%)
20 ~~10⁵ / ml~~ ~~25%~~	① - 38 ⑩ - 2
a.u. + x% / ml 26 ~~6 - 10⁵ / ml~~ ~~+ 25%~~	X

(11-LAS)

10^{-4}	5	14	20	26
$7 \cdot 10^5 \text{ m/s}$	38	$1 \cdot 10^5 \text{ m/s}$	$6 \cdot 10^5 \text{ m/s}$	$6 \cdot 10^5 \text{ m/s}$

many

[illegible]

$1V - 1A5 (9+5)$ 10^{-4}	14 	20 	26 
38 ρ''_{14}/m	26 	~~38 ~~	38 

$1.3 \cdot 10^4 \mu A$
 $(+25\%)$

$V - VEE$

[illegible]

VI - VEE

$\begin{array}{c} 3-14 \\ 10-9 \end{array}$	$\begin{array}{c} 20 \\ 26 \end{array}$	$\begin{array}{c} 38 \\ 38 \end{array}$
$\begin{array}{c} 4+7 \\ 10-4 \end{array}$	$\begin{array}{c} 0-5 \\ 10-4 \end{array}$	$\begin{array}{c} 0-5 \\ 10-2 \end{array}$

Handwritten notes and signatures are present in the right margin, including "Vee" and "10-2".

Inserted between

10/19/1991

IFA of infection 10/18 - a-lassa (~15 hrs p.i.)

I
LSN
878.3

12	24	36	48
0	0	0.2-10 ⁵	2.6-10 ⁵
12	24	36	48

12	24	36	48
0	0	0	0
12	24	36	48
0	0	0	0

12	24	36	48
0	0	0	0
12	24	36	48
0	0	0	0

10⁻³ dilutions~~Infection 10:30~~

10/20/1991

Large-scale plasmid DNA 3014 (~0.2 µg/µl)

3014 DNA cleavage 1) w/ Not I + BSA + ③ React

2) w/ Rsr II + Apa I + ⑤ React + BSA

Not I cleavage

3014 DNA 200 µl

React ③ 10x 80 µl

50 mg/ml BSA 1 µl

water 520 µl

Not I 3 µl

800 µl

at 37°C

Rsr II + Apa I cleavage

3014 DNA 200 µl

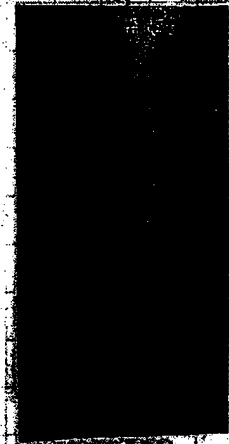
React ⑤ 10x 80 µl

50 mg/ml BSA 1 µl

water 520 µl

Apa I

Rsr II

800 µl
at RT

~ $0.5 \cdot 10^5$ (100%)
use undiluted

(10)
(11)
(12)
(13)

CSN+
PI-5

$10^{-5} \div 10^{-7}$

38h

5hr0

14hr0

20hr0

26hr0

A 097

2) BHK-22 cells trypsinized, 6 lattice slides covered at 10^5 cells per chamber, the rest of the (150 cells) transferred into new T25 flasks for splitting

3) DNA 1% TBE agarose 1) 3014/Not I/transcription grade 1 out of 70 μ l
2) 3014/Rsa I + Bpa I/cleavage 5 μ l
3) 1 KB MARKER

10/24/1994

1) 1% TBE Agarose 1) 3014/Not I/transcription grade 1 μ l out of 140 μ l
2) 3014/Rsa I + Apa I 5 μ l
3) KB marker

2) 3014/Rsa I + Apa I: 2x phenol-chloroform, precipitate, wash 3x 75% EtOH, dry, dissolve in 90 μ l water

10 μ l 10x T4 pol

3 μ l Mg^{2+} /NTP (30 mM total)

2 μ l T4 polymerase 5 u/ml

RT 20 min. 2x Phenol-chloroform, precipit, 3x 75% EtOH, dissol

1 μ l 1% Agarose/TBE

P5

2 μ l + 14 μ l water + 2 μ l buf + 2 μ l T4 DNA Lig 0/min +4°C

3) Electroporation BHK-23 (grown o/n after splitting)

① $V3000 = 272.3$ 20 μ l of 2.86% 5.72 μ g was tapped
 about 7 μ g in 30 μ l 10 μ l of 1.7% 1.7 μ g three times,
 5 μ l of 2.2% 1.1 μ g since first time
 35 μ l -8.542μ g time count = 0.0
 -1.22μ g
 -6.32μ g
 -7μ g

② LSN + P2-1
 LSN 8.6 μ g in 25 μ l
 P2-1 9 μ g in 25 μ l

③ $V3000 = 272.3$ 1.217 μ g in 5 μ l

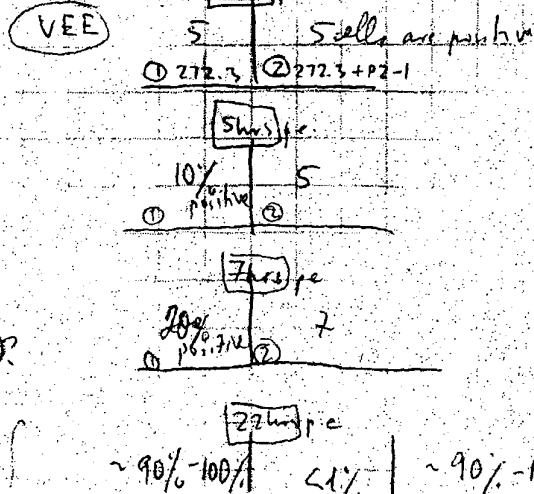
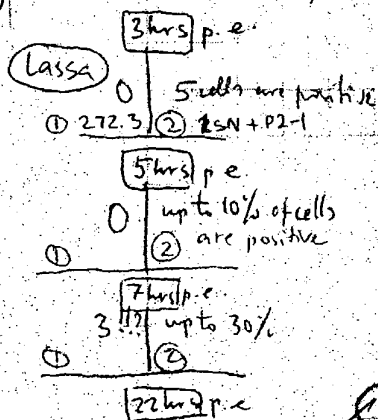
tapped cells have been transferred into 23 ml of medium, aliquots (400 μ l) were taken for IFA, cells w/ medium were transferred into 75 cm^2 flasks, incubation at 37°C 6% CO_2 started at 12:00

- Sample for IFA is taken at 15:00 (3 hrs p.e.)
- Sample for IFA is taken at 17:00 (5 hrs p.e.)
 - Sample for infectivity assay is taken at 17:00 (5 hrs p.e.)
- Sample for IFA is taken at 19:00 (7 hrs p.e.)
 - Sample for i.a. is taken at 19:00 (7 hrs p.e.)
- 22 hrs etc

10/25/1994

1) Plaque assay - counting see results in 10/23/94

2) IFA for LSN Ag and VEE



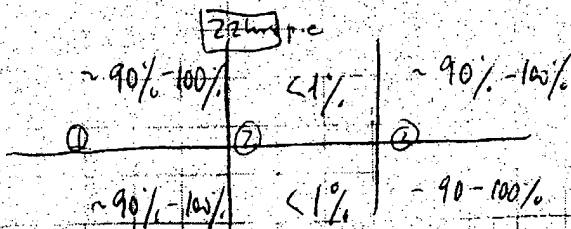
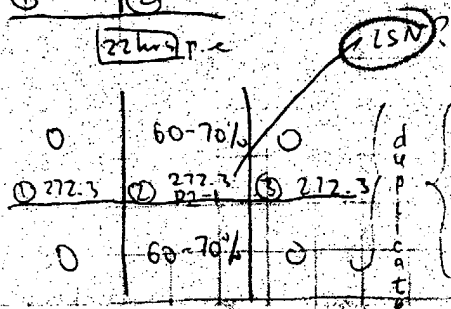
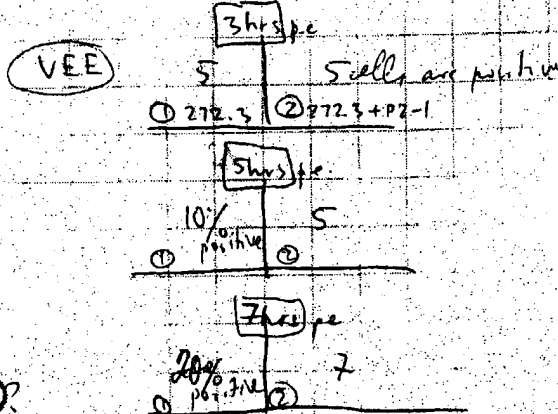
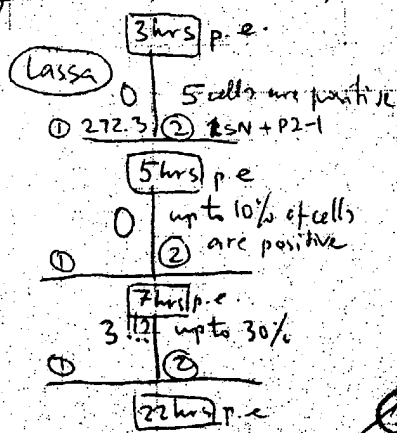
tapped cells have been transferred into 23 ml of medium, aliquots (400 μ l) were taken for IFA, cells w/ medium were transferred into 75 cm² flasks, incubation at 37°C 6% CO₂ started at 12:00

A 099

- a. Sample for IFA is taken at 15:00 (3 hrs p.e.)
- b. Sample for IFA is taken at 17:00 (5 hrs p.e.)
 - a. Sample for infectivity analysis is taken at 17:00 (5 hrs p.e.)
- c. Sample for IFA is taken at 19:00 (7 hrs p.e.)
 - b. Sample for i.a. is taken at 19:00 (7 hrs p.e.)
- d. 22 hrs etc

10/25/19

- 1) Plaque assay - counting (see results in 10/23/99)
- 2) IFA for LSN Ag and VEE



3) $\pm$ cDNA transformation P5

4) Infection of BHK cells

10/26/1994

1) ①	272.3 + LSN	5hr p.e.	undiluted sup.	Lassa Ag positive	total
2)	-	14 hrs	10^{-2}	0 (+10%)	0
3)	-	20	10^{-2}	10 (+50%)	$1.3 \cdot 10^5$
4)	-	20	10^{-3}	0 (+25%)	0
5)	-	26	10^{-3}	0 (+40%)	0
6)	-	38	10^{-3}	7 (+75%)	$1.2 \cdot 10^5$
7)	-	38	10^{-4}	2 (+80%)	$4 \cdot 10^5$
8)	-	14	undiluted	26 (+90%)	$3.4 \cdot 10^6$

2) ②	P1-5 + LSN	5hr p.e.	undiluted sup.	Lassa Ag positive	total
2)	-	14	10^{-2}	0 (+50%)	0
3)	-	20	10^{-2}	2 (+90%)	$4 \cdot 10^4$
4)	-	20	10^{-3}	2 (+90%)	$4 \cdot 10^4$
5)	-	26	10^{-3}	2 (+40%)	$3 \cdot 10^4$
6)	-	38	10^{-3}	2 (+50%)	$1.4 \cdot 10^4$
7)	-	38	10^{-3}	2 (+25%)	$3.3 \cdot 10^4$
8)	-	14	10^{-3}	2 (+90%)	$2 \cdot 10^6$

14hr 272.3 + LSN 5; 20; 26; and 38 hrs p.e.
positive cells

3) ③	272.3 + LSN	12 hrs p.e.	undiluted sup.	Lassa Ag positive	total
2)	-	24	$10^{-1} / (10^{-2})$	100 (+50%)	$2 \cdot 10^5$
3)	-	24	10^{-3}	0 (+60%)	0
4)	-	36	10^{-2}	2 (+50%)	$4 \cdot 10^4$
5)	-	48	10^{-3}	11 (+90%)	$2 \cdot 10^4$
6)	-	60	10^{-3}	13 (+80%)	$2 \cdot 10^4$
7)	-	85	10^{-3}	24 (+70%)	$4 \cdot 10^4$
8)	-	12	undiluted	10 (+10%)	$20 \cdot 10^4$

2) (P5) - many clones : uncalculated 16 2ml-cultures
(P5K) - few clones

3) Clearance 3014 DNA kpm 2

5)	—	—	26
6)	—	—	38
7)	—	—	38
8)	—	—	14

10-3	0	(+75%)	0
10-3	7	(+80%)	1.2
10-4	2	(+90%)	4.10
und 10	26	(+30%)	34

②	1) P1-S + LSN	shr p.e
	2) — —	14
	3) — —	20
	4) — —	20
	5) — —	26
	6) — —	38
	7) — —	38
	8) — —	38

undiluted exp.	Cassa Ag	punkte	
10	0	(+50%)	4
2	2	(+90%)	4
2	20	(+90%)	4.1
5	2	(+40%)	3.1
		(+50%)	1.4
		(+25%)	3.3
		(+90%)	2.1
		(+10%)	

45N 5; 20; 26; and 38 hrs p.e.

③	272.3 + LSN	72 hrs p.e
1)	—	24
2)	—	24
3)	—	36
4)	—	48
5)	—	60
6)	—	85
7)	—	12
8)	—	

	Class A	non five
10 ⁻¹ (undiluted sup)	0	(+50%) 1
10 ⁻¹ (10 ⁻²)	100	(+50%) 2
10 ⁻³	0	(+60%) 0
10 ⁻³	2	(+50%) 4
10 ⁻³	11	(+40%) 2
10 ⁻³	13	(+80%) 2
10 ⁻³	24	(+70%) 4
undiluted	10	(+50%) 2

2) (p5) - many clones : inoculated 16 2ml-cultures
(p5k) - few clones

A 101

3) Cleavage 3014 DNA kpm 2

50 μ l	10x Mg^{2+} Reagent
1 μ l	50 mg/ml BSA
20 μ l	3014 DNA
430 μ l	H ₂ O
3 μ l	Km 2

a-lassa

10/76/94

undil.	10	10	
+40% 1-5 10 ⁻²	+50% 1-14 10 ⁻²	+25% 1-20 10 ⁻²	+40% 1-20 10 ⁻³
+75% 1-26 10 ⁻³	+88% 1-38 10 ⁻³	+90% 1-38 10 ⁻⁴	+30% 1-14 undil
0	7	2	26

+50% 2-5 undil	+40% 2-14 10 ⁻²	+90% 2-20 10 ⁻²	+40% 2-20 10 ⁻³
+50% 2-26 10 ⁻³	+25% 2-38 10 ⁻³	+90% 2-38 10 ⁻⁴	+40% 2-14 many undil
7	27	10	

10/16 1	+50% 1-12 10 ⁻²	+50% 1-24 10 ⁻²	+60% 1-24 10 ⁻³	+50% 1-30 10 ⁻³
3	+40% 1-48 10 ⁻³	+80% 1-60 10 ⁻³	+70% 1-85 10 ⁻³	+50% 1-12 undil
	14	13	24	10

4

a-VEE

undiluted	2-5	2-20	2-20	2-20
2-28	2-38			
				+50%

inserted
at pg 76-77

10/27/94

- 1) mini-preps of P5 1÷16; cleavage with *Sph I* o/n
 was 1,2,8 - 1% gel (uncleaved)

- 2) 3014 *Kpn I* - 1% gel

(P6)

3014 *Kpn I* - 2 μ l10 x ~~Boehr~~ but 2 μ l

and

14 μ l

Ligase

2 μ l

4°C - o/n

10/28/94

- 1) cleavage of P5-1; 2; and 3014 with *Sma I*
 (P5-1) is OK = Δ *Rsr II* *Kpn I* of 3014

P5-1/P5-2/3014/K6

10/29/94

- 1) Maxi-prep isolation of DNA P5-1
 Cleavage with *Not I*

- 2) Transformation P6 (3014 Δ *Kpn I*) into *E. coli* 845d

10/30/94

- 1) inoculated 2-ml LB cultures P6 1÷16

- 2) P5-1/*Not* deprotonated (2x), precipitated (-70°C) o/n

10/31/94

- 1) P6 1÷16 cells are frozen, mini-preps, cleavage o/n at 37°C *Rsr II* *Kpn I*

- 2) P5-1/*Not* precipitated, washed 3x 75% EtOH, dissolved in 40 μ l water

P5-15 16 3014

- 1) 1% TBE Agarose gel: P6 1÷16 *Rsr II* *Kpn I*; P5-1/*Not I*

P5-1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

P5-1

OK
 Cleavage (P8-2): 1) Apa I Bst E II
 2) Apa I Kpn I

1% Agarose

2) TRANSCRIPTIONS

4 hrs at 37°C

	(LSN) OK	(3014) OK	(P5-1) OK
water	18.8	8.7	4.7
5x BUF	16	8	8
25mM ATP/G	24	12	12
25mM cap	9.6	4.8	8.4
20x A/Not I	1	1	1.5
H ³ UTP	1.3	0.7	0.7
100 mM GTP	1.4	0.8	0.7
10x Enzyme mix	8	4	4

11/2/94

1) checking yesterday's

LSN 4.14%

P5-1 4.79%

523-5075

2.8%

2) TRANSCRIPTION 3014 as in 11/1/94

11/3/94

1) checking transcription

3014: 0.7%

2) plaque assay

①	272.3/-5	$10^{-2} \div -4$	0
2	LSN+P2-1/-5	$10^{-2} \div -4$	0
* 3	272.3/-5	$10^{-2} \div -4$	0
④	272.3/-9	$10^{-2} \div -4$	2.10 ²
5	LSN+P2-1/-9	$10^{-2} \div -4$	0

⑩	272.3/-27	$10^{-4} \div -6$	6.10 ⁵
11	LSN+P2-1/-27	$10^{-2} \div -4$	0
* 12	272.3/-27	$10^{-4} \div -6$	8.10 ⁵
⑬	272.3/-31	$10^{-4} \div -6$	2.10 ⁵
14	LSN+P2-1/-27	$10^{-2} \div -4$	0

5X BUF 16
 25m MAMP/G 24
 25m M cap 9.6
 200A/NOT-I 1
 H3 UTP 1.3
 100 mM GTP 1.4
 10X Suzyne mix 8

8
 12
 4.8
 0.7
 0.7
 0.7

A 105

11/2/94

1) checking yesterday's

LSN 4.14%

523.5075

LSN 3014 P51

2) TRANSCRIPTION 3014 as in 11/1/94

11/3/94

1) checking transcription

3014: 0.7%

2) plaque assay

① 272.3/-5 $10^{-2} \div -4$ 0
 2 LSN+P2-1/-5 $10^{-2} \div -4$ 0
 * 3 272.3/-5 $10^{-2} \div -4$ 0
 ④ 272.3/-7 $10^{-2} \div -4$ $2 \cdot 10^2$
 5 LSN+P2-1/-7 $10^{-2} \div -4$ 0
 * 6 272.3/-7 $10^{-2} \div -4$ $6 \cdot 10^2$
 ⑦ 272.3/-22 $10^{-4} \div 10^{-6}$ $2 \cdot 10^5$
 8 LSN+P2-1/-22 $10^{-2} \div -4$ 0
 * 9 272.3/-22 $10^{-4} \div 10^{-6}$ $1.5 \cdot 10^6$

⑩ 272.3/-27 $10^{-4} \div -6$ $6 \cdot 10^5$
 11 LSN+P2-1/-27 $10^{-2} \div -4$ 0
 * 12 272.3/-27 $10^{-4} \div -6$ $8 \cdot 10^5$
 ⑬ 272.3/-31 $10^{-4} \div -6$ $2 \cdot 10^5$
 14 LSN+P2-1/-31 $10^{-2} \div -4$ 0
 * 15 272.3/-31 $10^{-4} \div -6$ $1.1 \cdot 10^5$
 ⑭ 272.3/-45 $10^{-4} \div -6$ $1.3 \cdot 10^5$
 17 LSN+P2-1/-45 $10^{-2} \div -4$ 0

* 18. 272.3/45 $10^{-4} \pm -6$ $1.2 \cdot 10^7$
 (19) 272.3/-70 $10^{-4} \pm -6$ $4 \cdot 10^5$
 20 LSN+P2-1/-70 $10^{-2} \pm -4$ $\square$
 * 21 272.3/-70 LSN p.o. $10^{-4} \pm -6$ $6 \cdot 10^6$

- 1) Maxi-Prep Inactivation of P6-2 (2014 & KymL) 10/4/94
 Cleavage w/NotI: 800 μ l water, 100 μ l P6-2, 100 μ l 10x R(3), 2 μ l 50 mg/ml
 BSA; 4 μ l 100 μ l NotI (BRL) 12^h
 2) Plaque assay (cont'd)

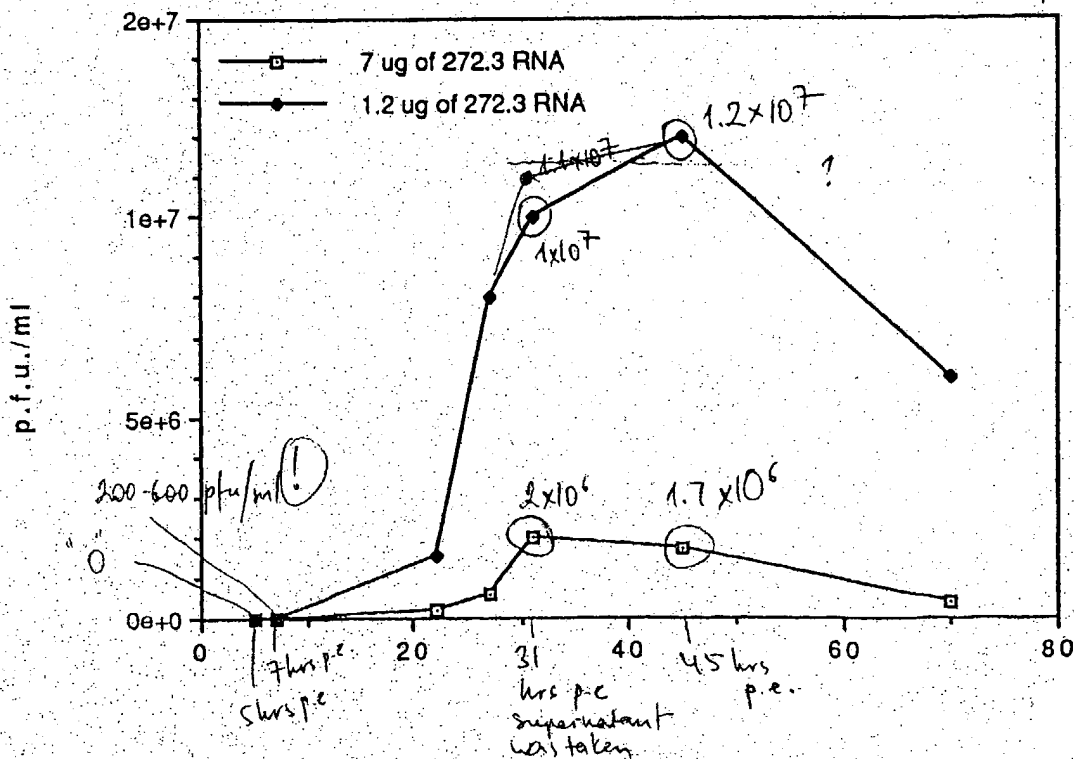
peter-11/3/94
 LSN+P2-1

p.e., hours	272.3-7 ug	272.3-1.2 ug		
1	5.000	0.000	0.000	0.000
2	7.000	200.000	600.000	0.000
3	22.000	200000.000	1500000.000	0.000
4	27.000	600000.000	8000000.000	0.000
5	31.000	2000000.000	10000000.000	0.000
6	45.000	1700000.000	12000000.000	0.000
7	70.000	400000.000	6000000.000	0.000

Cells also were f.p.w
 VEE AR
 after e.p.
 P2-1-dead?

BHK transfection with 272.3 RNA

plaque assay



16/5/1994

TRANSCRIPTION

3014/Not I

P6-2/Not I

Water	8.7
5x buf	8
25mM NTP/-G	12
25mM Cap	4.8
8NA	0.9
4x UTP	0.7
100 mM GTP	0.8
10x Enz mix	4

8.8
8
12
4.8
1
0.7
0.7
4

37°C 16:00 - 19:10

11/6/94

1) unselected

TRANSCRIPTION -

Not counted!

2) TRANSFECTION

1) 3014

15/4.79 + 15/0.7 + 15/1

2) 3014 <

2 μ l/4.79 + 2 μ l/0.7 + 2 μ l/1

3) LSN/3014

15/4.79 + 15/0.7 + 15/1

10/3.59 + 15/4.1

4) LSN/PS-1

18 ~~20~~/4.8

10/3.59 + 15/4.1

5) PS-1

18 ~~20~~/4.2

6) (LSN) P6-2

18/1

10/3.59 + 15/4.1

7) P6-2

18/1

8) NC (just cells (electroporated)) -

1130 incubation began

5x buf
25 mM NTP/-G
25 mM cap
80A
43 UTP
100 mM GTP
10x Enz mix

8
12
4.8
0.9
0.7
0.8
4

8
12
4.8
1
0.7
0.7
4

A 108

37°C 16:00 - 19:10

11/6/94

1) unisystem TRANSCRIPTION - checking
not counted

2) TRANSFECTION

1) 3014 $\frac{15}{4.79} + \frac{15}{0.7} + \frac{15}{?}$

2) 3014 $\frac{2 \mu l}{4.79} + \frac{2 \mu l}{0.7} + \frac{2 \mu l}{?}$

3) LSN/3014 $\frac{15}{4.79} + \frac{15}{0.7} + \frac{15}{?}$

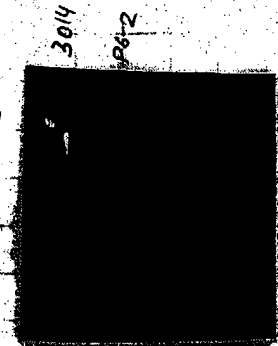
4) LSN/PS-1 $\frac{18}{4.8}$

5) PS-1 $\frac{18}{4.8}$

6) (LSN) P6-2 $\frac{18}{?}$

7) P6-2 $\frac{18}{?}$

8) NC (just cells (electroporated) -



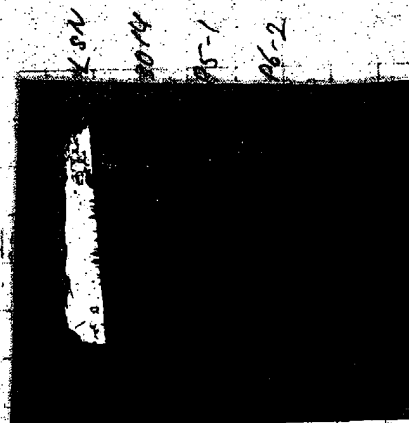
11:30 incubation began

Cells did not attach to surface. Caused by infection?
bacterial

11/7/94

TRANSCRIPTIONS

	LSN	3014	P5-1	P6-2
water	18.8	8.7	4.7	8.8
5x Buf	16	8	8	8
25mM NTP/-G	24	12	12	12
25mM cap	4.6	4.8	8.4	4.8
8NA/Not I	1	1 (0.91)	1.5	1
43 UTP	1.3	0.7	0.7	0.7
100 mM GTP	1.4	0.8	0.7	0.7
10x Euzyme	8	4	4	4
	7.7%	3.3%	4.8%	4.9%



11/8/94

TRANSCRIPTIONS

	3014	LSN	P6-2
water	8.7	8.8	8.8
5x Buf	8	8	8
25mM NTP/-G	12	12	12
25mM cap	4.8	4.8	4.8
8NA/Not	0.9	0.5	1
43 UTP	0.7	0.7	0.7
100 mM GTP	0.8	0.7	0.7
10x Euz mix	4	4	4
	2.4%	5.9%	5.7%

82

11/9/94

Checking BHK cells

11/8/94 cells were ~ healthy, ~ 80% confluent, 0%

11/9/94 cells contain granules + overgrowth, many dead cells in medium, 70% confl. (Mike)
 not confluent (Landy), more than confluent (George)
 trypsinization slow,
 suspended with difficulties

1 ml of trypsinized cells in stop solution (medium) +
 1 ml of Trypan blue
 count cells in hemacytometer,
 2 out of 64 are dead (blue),
 overall amount of cells $2 \cdot 10^7$ per +150 flask

Cells prepared as usually for e.poration

0.8 ml of cells e.porated w/o RNA

0.8 ml of cells not e.porated

Seeded into 75 cm² flasks 11:20

attached cells: 13:20 ~ 30% of surface

TRANSFECTIONS:

- ✓ 1) 3014 10/8.4 + 11/2.3 ✓ 3) 10/5.9 + 10/4.14
 3014 + LSN
 2) 3014 1/8.4 + 1/3.3 ✓ 4) 90/4.79 + 10/1.14
 P5-1 + LSN 10/5.9 + 10/4.4

Seeded into 75 cm² flasks 15:00

supernatant

T = A

- 1) 5 hrs 30 min p.e. 1) 5 hrs p.e.
 2) 7 hrs 30 min p.e. 2) 7 hrs 30 min p.e.
 3) 19 hrs p.e. 3) 19 hrs p.e.
 4) 27 hrs p.e.
 5) 41 hrs p.e.
 6) 90 hrs p.e.

IFA:

11/10/94

α -Lassa conjugate: 5 hrs p.e.

1) sample	0 positive
2)	0 positive
3)	(few) positive
4)	(0-1%) of cells are positive (many dead)

7 hrs p.e.

1)	0
2)	0
3)	(~3%) positive
4)	(~5%) positive

19 hrs p.e.

1)	0
2)	0
3)	(~5%) positive
4)	(~90%) positive

!!!

 α -VEE

5 hrs p.e.

1)	50% positive
2)	(10%)
3)	(~20%)
4)	** ~1%

7 hrs p.e.

1)	90%
2)	(50%)
3)	(90%)
4)	** ~5%

19 hrs p.e.

1)	100%
2)	(100%)
3)	(100%)
4)	** ~20% !!!

Remarks: synthesis of Lassa Ag starts in about 5 hrs in these cells (in earlier experiment, in about 3 hr: does it depend on helper virus or quality of cells?)

Synthesis of VEE Ag starts earlier than in 5 hrs p.e. (up to 10% of cells synthesize VEE Ag in 5:30 p.e.)

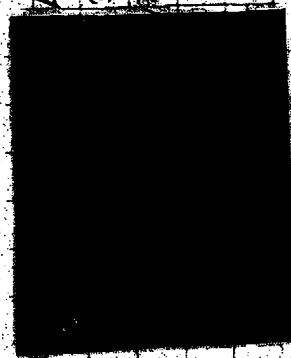
In 7 hrs p.e. expression of LSN Ag was similar in (LSN+3014) and (LSN+P5-1) cells, but in 19 hrs p.e. there were 5% positive for LSN-Ag in (LSN+3014) and

84

TRANSCRIPTIONS

	LSN	3014	P5-1
water	8.8	8.7	4.9
5x buf	8	8	8
25mM NTP/-G	12	12	12
25mM caps	4.8	4.8	8.4
RNA/NOT	0.5	0.9	1.3
H ³ ATP	0.7	0.7	0.7
100 mM GTP	0.7	0.8	0.7
RTX 2mg	4	4	4

LSN (44%)
3014 (8.3%)
P5-1 (14%)



LSN 0.5 µg/µl
3014 0.1 µg/µl
P5-1 0.3 µg/µl

10/14/94

3014, LSN, P1-5, and P6-2 RNAs are combined from different transcription reactions, concentration of RNA determined by gel electrophoresis and scintillation counting

LSN 15.6%

3014 4.2%

P5-1 10.9%

P6-2 4.3%

(0.3%)

LSN ~ 0.8 µg/µl ~ 80 µl
3014 ~ 0.2 µg/µl ~ 80 µl
P5-1 ~ 0.6 µg/µl ~ 80 µl
P6-2 ~ 0.1 µg/µl ~ 70 µl

(*)
(*)
(*)
(*)

NC 0

TRANSCRIPTIONS

	LSN	P5-4	P6-2
water	8.8	4.7	8.8
5x buf	8	8	8
25mM ATP/-G	12	12	12
25mM cap	4.8	8.4	4.8
DNA/Not	0.5	1.5	1
H3 ATP	0.7	0.7	0.7
100mM GTP	0.7	0.7	0.9
10x 2u2	4	4	4

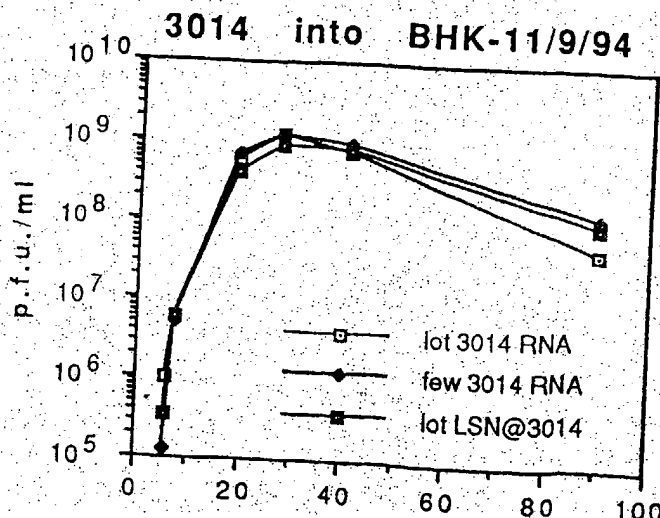
{ LSN 38% ?

{ P5-1 35% ?

{ P6-2 32% ?

LSN	0.3 mg/ml	~ 35 µl
P5-1	0.3 mg/ml	~ 35 µl
P6-2	0.2 mg/ml	~ 35 µl

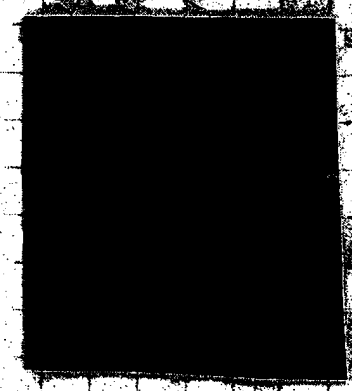
3014 into BHK-11/9/94



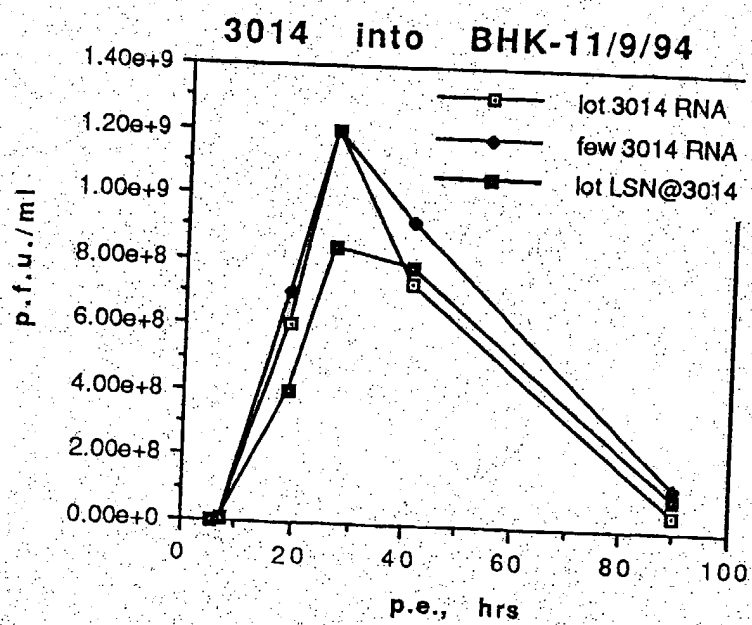
176.42x10 ²	④	undil	44	0.0
276.84x10 ²	④	undil	90	0.0
41.673x10 ²	3	NC undil	23	0.0
41.672x10 ²	4	NC undil	31	0.0
41.678x10 ²	5	NC undil	44	0.0
70.45x10 ²	②6	10/13 p1-5 undil	5hr	0
70.13x10 ²	27	undil	14hr	0
70.1x10 ²	28	undil	26hr	0
50	29	undil	26hr	0

8.8	4.7	8.8
8	8	8
12	12	12
4.8	8.4	4.8
0.5	1.5	1
0.7	0.7	0.7
0.7	0.7	0.9
4	4	4

} LSN 38% ?
 } PS-1 35% ?
 } PG-2 32% ?



LSN 0.3 mg/ml ~ 35 pp
 PS-1 0.2 mg/ml ~ 35 pp
 PG-2 0.2 mg/ml ~ 35 pp



27	undil	44	0
28	undil	90	0
41	undil	23	0
41	undil	31	0
41	undil	44	0
90	undil	5hr	C
70	undil	14hr	0
70	undil	20hr	0
50	undil	26hr	0
10	undil	38hr	0

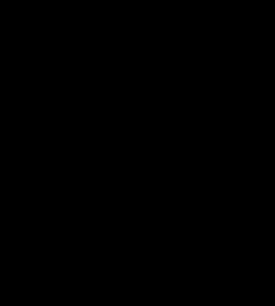
26 ÷ 30 e.p. 10/13/94
 1 ÷ 25 e.p. 11/9/94

W 0.6	8.8	4.7	8.8
5.6	8	8	8
2.5 7/G	12	12	12
Cap	4.8	8.4	4.8
Not	0.5	1.5	1
ASP	0.7	0.7	0.7
MM GTP	0.7	0.7	0.9
W 2uz	4	4	4

*4506, 740

15125

5304. 13



① undisturbed c.s. $10^{-2} \pm 10^{-4}$ 18×10^5 ② 55

p.e., hrs lot 3014 RNA few 3014 RNA lot LSN@3014

1	5.500	1000000.000	130000.000	350000.000
2	7.500	6000000.000	5000000.000	6000000.000
3	19.000	00000000.000	00000000.000	00000000.000
4	27.000	00000000.000	00000000.000	40000000.000
5	41.000	30000000.000	20000000.000	80000000.000
6	90.000	45000000.000	30000000.000	00000000.000

20 1.2 x 10 ²	undil	44	0
20 1.5 x 10 ²	undil	90	0
20 2.3 x 10 ²	NE undil	23	0
41 4.2 x 10 ²	NE undil	31	0
41 6.7 x 10 ²	NE undil	44	0
90 4.5 x 10 ²	10/13 ^{ice} 11-5 undil	5hr	0
90 1.3 x 10 ³	undil	14 hr	0
90 1 x 10 ³	undil	20 hr	0
5 0	undil	26 hr	0
1 0	undil	38 hr	0

26 ÷ 30 e.p 10/13/14
1 ÷ 25 e.p 11/9/94

1% Agarose: pV3000 (0.5 μ l); 1 Kb
 concn. $\sim$ 1 μ g/ μ l

11/17/94 TRANSFECTIONS CEF cells (seeded yesterday)

✓ 1. 3014 (30 μ l) $\sim$ 6 μ g

✗ 2. 3014 (2 μ l) $\sim$ 0.2 μ g + 25 μ l UH

RNA from 11/14/94 *
*
*

✓ 3. LSN + 3014 (30 μ l) $\sim$ 6 μ g
 (3 μ l) $\sim$ 10 μ g

next time take 2×10^7 cells/ml

✓ 4. LSN + 3014 (1 μ l) $\sim$ 0.2 μ g
 (3 μ l) $\sim$ 10 μ g

10^7 cells/ml

✓ 5. LSN + P5-1 (10 μ l) $\sim$ 10 μ g

normal cells + RNA

seeded into ~~25~~ 25 cm^2 flasks

6. LSN + P6-2 (7 μ l) $\sim$ 4 μ g
 (3 μ l) $\sim$ 10 μ g

CEF in 5 hrs p.e. seem to be adhered to plastic but many cells are rounded, as they are not very well attached.

7. P6-2 (47 μ l) $\sim$ 4 μ g

25 cm^2 flask seems to be just OK for 10^7 cell/ml for CEF (better take 2×10^7 cells)

8. NC (w RNA)
 1/2 known

18:00 for IFA-transfection eff.

- 1) 5 hrs p.e.
- 2) 14 hrs p.e.

for Plaque assay and i.u.

- + 1) 6 hrs p.e. (0%)
- + 2) 14 hrs p.e. (8%)
- + 3) 22 hrs p.e. (16%)
- + 4) 30 hrs p.e. (100%)
- + 5) 38 hrs p.e. (8%)
- + 6) 46 hrs p.e. (16%)

11/17/94 TRANSFECTIONS (CEF cells) (seeded yesterday)

✓ 1. 3014 (30 μ l) ~ 6 μ g✗ 2. 3014 (2 μ l) ~ 0.2 μ g + 25 μ l UN

RNA from 11/14/94

✓ 3. LSN + 3014 (30 μ l) ~ 6 μ g
(3 μ l)
~ 10 μ gnext time have 2×10^7 cells/ml✓ 4. LSN + 3014 (1 μ l) ~ 0.2 μ g
(3 μ l)
~ 10 μ g 10^7 cells/ml✓ 5. LSN + P6-2 (17 μ l)
(3 μ l)
~ 10 μ g

cells + RNA

seeded into ~~25~~ 25 cm^2 flasks6. LSN + P6-2 (17 μ l)
(3 μ l)
~ 10 μ g ~ 4 μ g

CEF in 5 hrs p.e. seem to be adhered to plastic but many cells are rounded, as they are not very well attached.

7. P6-2 (47 μ l)
~ 4 μ g25 cm^2 flask seems to be just OK for 10^7 cell/ml for CEF (better take 2×10^7 cell).8. NC (w RNA)
1/2 known

18:00 for IFA-transfection eff. for plaque assay and i.u.

1) 5 hrs p.e.
2) 14 hrs p.e.

+ 1)	6 hrs p.e.	(0 ^u)
+ 2)	14 hrs p.e.	(8 ^u)
+ 3)	22 hrs p.e.	(16 ^u)
+ 4)	30 hrs p.e.	(00 ^u)
+ 5)	38 hrs p.e.	(8 ^u)
+ 6)	46 hrs p.e.	(16 ^u)
+ 7)	54 hrs p.e.	(16 ^u)
+ 8)	70 hrs p.e.	(16 ^u)

11/18/94

IPA

1) 5 hrs p.e.

	a-Lassa	a-VEE
1. 3014 (6 μ g)	"0"	20-30%
2. 3014 (0.2 μ g)	"0"	few
3. LSN + 3014 (6 μ g)	few	20-30%
4. LSN + 3014 (0.2 μ g)	few	few
5. LSN + PS-1	few	few
6. NC	"0"	"0"

2) 14 hrs p.e.

	a-Lassa	a-VEE
1. 3014 - 6 μ g "0"	100%	
2. 3014 - 0.2 μ g "0"	90-100%	
3. LSN + 3014 (6 μ g) 30-40%	100%	
4. LSN + 3014 (0.2 μ g) 30-40%	90% - 100%	
5. LSN + PS-1 30-40%	~10-20%	
6. NC "0"	"0"	

Generally, cells are poorly attached to plastic 5 hrs p.e., many dead cells. Cells look much better, better attached to plastic in 14 hrs p.e.

Probably, need to use more LSN RNA for transfection (and PS-1?). RNA amount (*) is too high, in reality probably 300 ug/ μ l LSN

Generally, synthesis of either LSN A or VEE begins in 5 hrs p.e. (few positive cells), may be a little bit slower than in 18 hrs cells.

Improvements of CEE use: 1) use more LSN RNA (3x more)
2) take more cells (2 x 10⁷ cells/ μ l)
3) make IPA ~ 20 hrs p.e.

11/21/44

18hr-79 cells (mice) were trypsinized
and seeded at 80,000/chamber of
8-chamber Lab-Tec slides 10:30 am
(for i.u. analysis)

at 16:30 they are still
very poorly attached to plastic - rounded,
clumped etc. (!?)

Clearance o/n at 37°C 5014 : Apr 1 + Apr 11

3014 SNA	50
10x RACT	50
Apr 1 & Apr 11	some
water	400

pV3000 is inoculated into LB-broth (2ml) o/n at 37°C shaking

11/22/44

To B. Johnston

SNA P5-1	100 μ l
NH ₄ Ac	15 μ l
Ethanol	300 μ l

-20°C and to John Smith

I.U. analysis
Lab-Tec slide ①

27 hrs p.e.			30 hrs p.e.
11/4 ①	11/4 ③	11/4 ④	11/7 ①
conc 0	10^{-4}	10^{-4}	conc 0
	43	35	
11/7 ③	11/7 ④	11/7 ⑤	nc
10^{-4}	10^{-4}	10^{-4}	
3	0	0	0
②			
11/4 ①	11/4 ③	11/4 ④	11/7 ①
conc 0	10^{-5}	10^{-5}	conc 4
	3	4	
11/7 ③	11/7 ④	11/7 ⑤	nc
10^{-5}	10^{-5}	10^{-5}	
0	0	1	0

blue cross
amount/number
of possible (SNA)

(43) taken with
factor of dil
and 100 μ l
infection
med
4.3 $\times 10^6$ i.

clumped etc. (1-)

A 120

Clearance of n at 37°C 3014 : Apr I + Apr II

3014 DNA 50
 10x ReACT 50
 Arr I & Apr I some
 water 400

pV3000 1) inoculated into LB-bmp (2ml) of n at 37°C shake

11/22/94

To B. Johnston:

DNA P5-1 100 ~~µl~~
 NH₄ Ac 15 ~~µl~~
 Ethanol 300 ~~µl~~

-20°C and to John Smith

I.U. analysis
 laster slide ①

27 hrs p.e.			30 hrs p.e.
11/9 ①	11/9 ③	11/9 ④	11/17 ①
conc 0	10 ⁻⁴ 43	10 ⁻⁴ 35	conc 0
11/17 ③	11/17 ④	11/17 ⑤	nc
10 ⁻⁴ 3	10 ⁻⁴ 0	10 ⁻⁴ 0	0
②	11/9 ①	11/9 ③	11/9 ④
conc 0	10 ⁻⁵ 3	10 ⁻⁵ 4	conc 0
11/17 ③	11/17 ④	11/17 ⑤	nc
10 ⁻⁵ 0	10 ⁻⁵ 0	10 ⁻⁵ 1	0

blue shows
 amount of num
 of positive (LS)

④, taken with
 factor of 0
 and 100 µl
 infection
 means
 4.3 x 10⁶

1 hr at 37°C infection w/ 100 µl of medium + supernatant
 at appropriate dilution, + 300 µl of medium at 37°C
 15:00 - 8:00

11/25/94 Plaque assay from transfection 11/17 virus in chick cells

1 ① - 6 hrs p.e. 5×10^5	11 ② - 22 7×10^6	21 ③ - 38 1.5×10^7
2 - 14 hrs 7×10^6	12 30/14 - 30 1.1×10^7	22 - 46 8×10^6
3 - 22 hrs 8×10^6	13 30/14 - 38 1.3×10^7	23 - 54 6×10^6
4 - 30 hrs 1.5×10^7	14 30/14 - 46 1.3×10^7	24 - 70 3×10^6
5 30/14 - 38 hrs 1.8×10^7	15 30/14 - 54 1×10^7	25 ④ - 6 4×10^4
6 30/14 - 46 hrs 6×10^6	16 30/14 - 70 7×10^6	26 30/14 - 14 1×10^6
7 30/14 - 54 hrs 1.6×10^7	17 ③ - 6 3.8×10^5	27 30/14 - 22 3×10^6
8 - 70 hrs 3×10^6	18 30/14 - 14 4×10^6	28 30/14 - 30 5×10^6
9 ② - 6 2×10^4	19 30/14 - 22 5×10^6	29 30/14 - 38 4×10^6
10 14 5×10^6	20 30/14 - 30 7×10^6	30 30/14 - 46 6×10^6
		31 30/14 - 54 5×10^6
		32 - 70 1.3×10^7

LSN+ { ⑤ - 30 hrs 33
PS-1 { ⑥ - 38 hrs 0
NC { ⑥ - 30 hrs

00031 only
11/25/94

Clearance virus. AggIRs + continued
Virus cells trypsinized & split into 4 150 flask and 6 25 flasks

11/26/94 ① TRANSFECTION CHIC 2×10^7 cells/ml 14:00

- 11/7 15 μ l marked 11/10, gel on 11/14 15 μ l
- 1) LSN + PS-1 15 μ l
- 2) LSN + PS-2 25 μ l marked, gel on 11/14 11/7 25 μ l

plus Spl from 10/9
(LSN
PS-1
PS-2)

3) PS-2 (25 μ l) gel on 11/14

0.8 ml + 10 ml Comp medium
into 25 cm² flasks

Table

Analysis of the supernatants of BHK cells transfected with full length; and replicon and/or helper RNAs

BHK cells	Helper	LSN Rep	i.u./ml, LSN	i.u./ml, VEE	p.f.u./ml
	-	+	<100	n.t.	n.t.
272.3full length	-	-	n.t.	n.t.	1.2x10exp7
272.3full length	+	+	4-9x10exp5	n.t.	4x10exp6
272.3dRsrApa, P1-5	-	-	<100	• <10	<100
272.3dRsrApa, P1-5	+	+	8-20x10exp5	• <100	<100
272.3dKpn, P2-1	-	-	n.t.	n.t.	n.t.
272.3dKpn, P2-1*	+	+	n.t.	n.t.	n.t.
3014full length	-	-	<10	n.t.	1.2x10exp9
3014full length	+	+	4x10exp6	n.t.	8.4x10exp8
3014dRsrApa, P5-1	-	-	n.t.	n.t.	n.t.
3014dRsrApa, P5-1	+	+	4x10exp6	n.t.	<100
3014dKpn, P6-2	-	-			
3014dKpn, P6-2	+	+			

* does not function as helper, since no synthesis of VEE structural proteins was detected after electroporation with both RNAs, as determined by IFAs (for LSN Ag and VEE). Why it doesn't ????

CEF cells

Helper	LSN Rep	i.u./ml, LSN	i.u./ml, VEE	p.f.u./ml
3014full length	-	n.t.	n.t.	2x10exp7
3014full length	+	7x10exp4	n.t. <i>confirmed</i>	2x10exp7
3014dRsrApa, P5-1	-	n.t.	• n.t.	n.t.
3014dRsrApa, P5-1	+	2x10exp5	• n.t.	<100
3014dKpn, P6-2	-			
3014dKpn, P6-2	+			

So at 10^{-3} and 10^{-4} dilution

5 hr p. infection

(3)

RNA pV3000/ApaI+LsrII
 Water
 10x T4 pol
 dNTP
 T4 pol

10 μ l
~~75 μ l~~
 10 μ l
 3 μ l
 2 μ l

20 min RT
 twice phenol-chloro
 precipitation

(4)

RNA pV3000 10
 10x React 5 10
 U₁₂ 80
 KpnI 800
 0/h at 37°C

RNA 3014 10
 10x (5) 10
 U₁₂ 80
 LsrII 2 μ l / 37°C

11/28/94

Cleavage OK, phenolized 1x, precipitated

TRANSCRIPTION

LSN

PS-1

as in

4/14

LSN1
 LSN2
 PS-1

24071.68

LSN1

4841.685

14908.91

LSN2

46%

PS-1

53%

NC

0.6%

Ligations

P7: pV3000 / RsrII/ApaI/T4

0

DNA 2 μ l

P8: pV3000 / KpnI

many
colonies10x ligation buffer 2 μ lH₂O14 μ l

P9: pV3000 / KpnI/T4

0

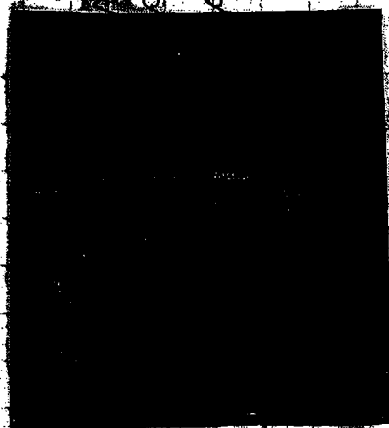
Cipase

2 μ l

P10: pV3014 / RsrII/SpeI/T4: 10 colonies

O/N at 4°C

P7 (RsrII/ApaI/T4)
 P8 (KpnI)
 P9 (KpnI/T4)
 P10 (RsrII/SpeI/T4)



-AH!!!

! Double band!!

11/29/95

1) P10 phenolized, precipitated, cleaned w/KpnI
 aliquot - BSA & CS₂

2) TRANSFORMATION

3) RNA extraction from → 1) 3014 - ① - chick transferrin "1/17
 3 × 10⁶ pfu/ml → 2) P5-12 LSN - ⑤
 3 × 10⁵ pfu/ml → 3) P1-52 LSN
 10⁶ pfu/ml

RNA dissolved in 70 μ l → -70°C

130/94 1) The rest of P7, P8, P9, P10 → Amp plates

- 2) a. LSN plasmid { 2ml LB-Amp^r inoculated
 b. 3026 ---
 c. P8 1:14 inoculated and P10 1:10 (volumes) inoculated

3) TRANSFECTION

① LSN & P5-1 (30μl + 30μl)

② LSN & P5-1 (15μl + 15μl)

③ LSN & P5-1 (5μl + 5μl)

④ LSN & P6-2 (15μl + 15μl)

⑤ P5-1 (15μl)

⑥ P6-2 (10μl)

75cm² flasks (20ml)

1120 - 1420 (27 hrs p.e.)

12/01/94

IFA - a-lasse and a VEE (TRANSFECTION EFF.)
 22 hrs p.e.

a-lasse

① ~100%	② ~100%	③ ~100%
④ ~100%	⑤ ○	⑥ ○

a-VEE

① ~100%	② 100%	③ 100%
④ ~70~80%	⑤ ○	⑥ ○

Mini-preps

P10 1:10

P8 1:14



[illegible]

1) or ? scht in November, 1944
by Nancy Sans I bet four.

Q - Lassa

① LSN & PC-1 (11/30/94) 27 hrs. v. c.

②

③

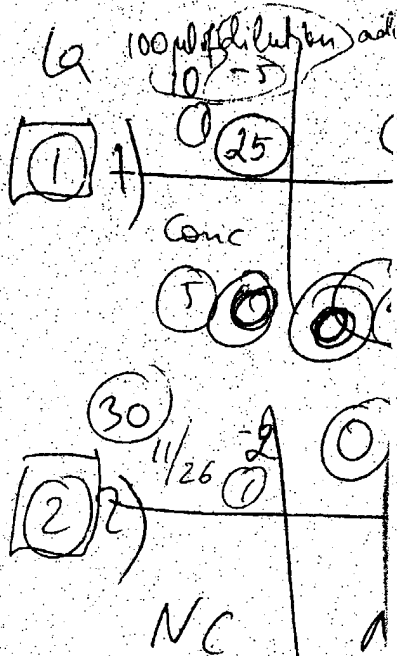
④

12/4/94

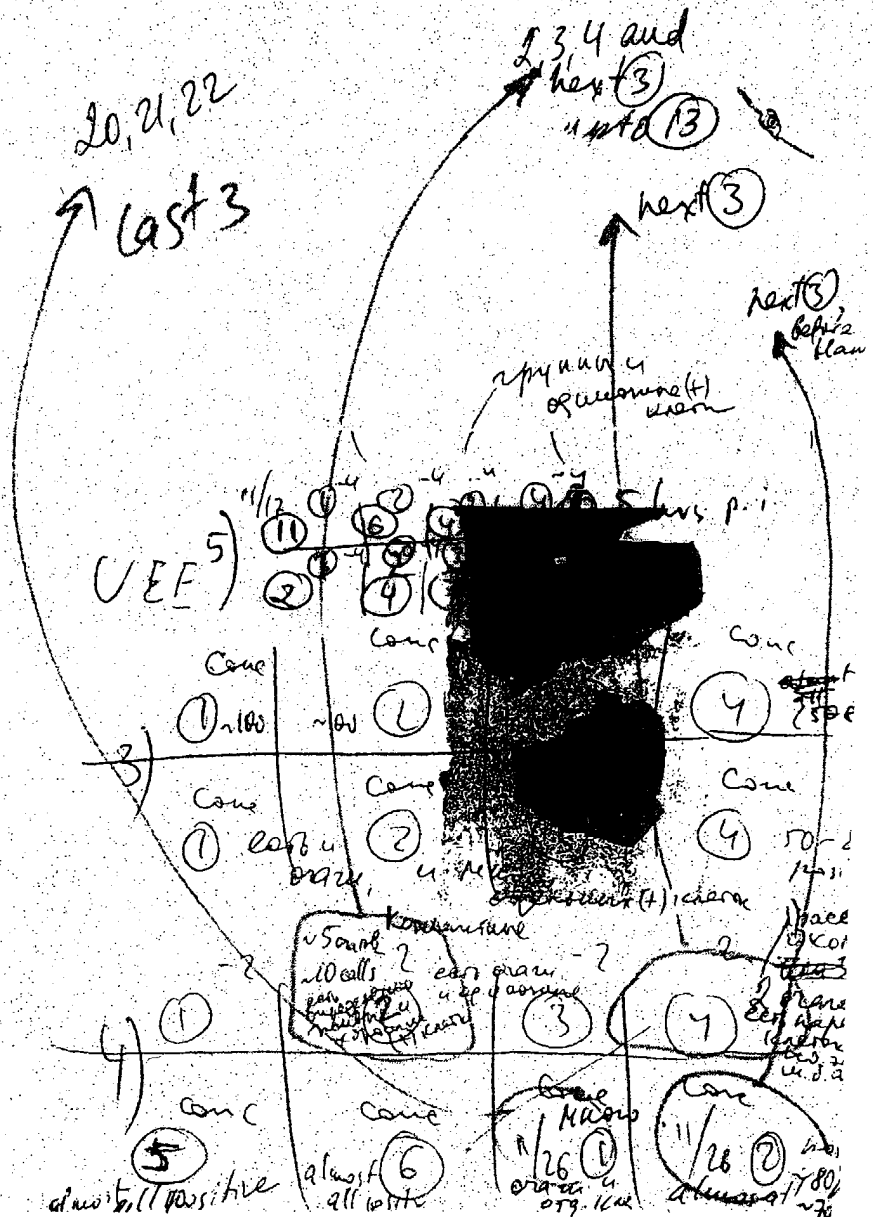
7 TEMPLATES FOR IN VITRO TRANSCRIPTION

- ① P8-1/Not I 1 μ l (out of 12) pV3000 Kpn I
- ② P10-1/Not I 1 μ l (out of 12) pV30145 Rsr II - Spt I
- ③ pV3000/Not I 1 μ l (out of 12) full length

174 494-49 & 495-49



a-VEE IFA



12/5/94

12/5/1994

Plaque assay

1 } transfection	11/26/94	$10^{-2} \div 10^{-7} \sim 3 \times 10^3$	11 } ② 11/26/94	$10^{-2} \div 10^{-7}$	1 plaque int 10^{-2} 100? or 0?
2 } ② 11/30/94			12 } ② 11/26/94		
3 } ② 11/30/94	-	7×10^2	13 } ③ 11/26/94	$10^{-2} \div 10^{-7}$	0 plaques
4 } ② 11/30/94	-		14 } ③ 11/26/94		
5 } ⑤ 11/30/94	-	1×10^3 ~~total~~	15 } ② 11/26/94	$10^{-2} \div 10^{-7}$	7×10^3
6 } ⑤ 11/30/94	-	1×10^7	16 } ② 11/26/94		
7 } ⑥ 11/30/94	-	~~transfection~~	17 } ④ 11/30/94	$10^{-2} \div 10^{-7}$	7×10^2
8 } ⑥ 11/30/94	-	7×10^6	18 } ④ 11/30/94		
9 } ① transfection	11/26/94	$10^{-2} \div 10^{-7}$	19 } ① 11/26/94	$10^{-2} \div 10^{-7}$	0 plaques
10 } ① 11/26/94		0 plaques	20 } ① 11/26/94		

IN VITRO TRANSCRIPTION

all as P8-1 ($\Delta 1404$)
 LSN in 11/14 P10-1 ($\Delta 3438$)
 P6-2 ($\Delta 1404$)
 pV3000 except

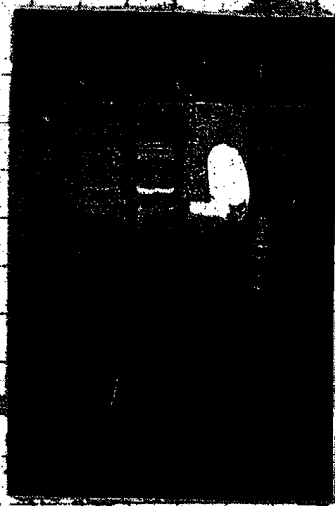
BNA were added 1 μ l (P6-2 - 1 μ l)
 and 0.4 100mM GTP was
 added to pV3000 and P6-2



12/6/94

Plasmid DNA

- 1) p2-20
- 2) LSN
- 3) 3526



TRANSCRIPTION

- 1) LSN
- 2) LSN
- 3) P5-8
- 4) V3000

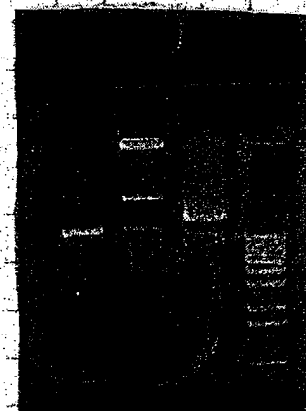
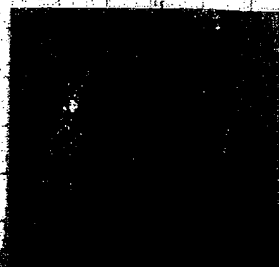
	LSN	P5-8	V3000
water	9.5	9.5	9.3
5x buf	8	8	8
25mM NTR/G	12	12	12
25mM cap	4.8	8.4	7.8
80A/Not	0.5	1.5	3.5
100mM GTP	0.7	0.2	0.9
Wx Ez	4	4	4

4 hrs 37°C

12/7/94

- 1) Checking transcription reactions

- 2) ClaI - cleavage of p2-20; LSN-plasmid; 3526



7/7/94 TRANSFECTIONS 16th1) 12/6 12/6
LSN + P5-1

20+25

2) 12/6 12/5
LSN + P10-1

20+25

3) 12/5
P5-1 204) 12/5
P10-1 20

5) 30/4

6) NC (No RNA)

11/8/94 TRANSFECTION EFFICIENCY 17 hrs p.e.

①	②	③
100%	100%	"0"
⑦	⑤	⑥
"0"	"0"	"0"

① and ② - practically,
100% of cells are
LSN-positive. 80%
of cells in culture③ & ④ - practically
100% negative, but
there are few "positive"
cells with probably
come from ① and ②⑤ and ⑥ - negative,
few "positive" cells,
as in ③ & ④, may
come from positive
① and ②

a - VEE
(17 hrs p.e.)

①	②	③
100%	100%	0 (?)
⑦	⑤	⑥
0	100%	0

INFECTIVITY ANALYSIS OF MEDIA

(infection in CalTEX slides w/100 μ l of
appropriate dil, then at 39°C + 300 μ l warm med
analysis 13 hr

a - Ca

10 ⁻⁵ ①	10 ⁻⁵ ②	conc ③	conc ④
25	9	0	0
conc ③	conc ⑥	10 ⁻⁴ ①	10 ⁻⁴ ②
9	0	254	91

2.8 x 10⁷ i.u./ml for LSN-Ag
0.9 x 10⁴ i.u./ml

a - VEE

①	②	③	④
conc some are small and hard to see	conc about 10 small and hard to see	conc 100% (+)	conc 6% are positive
10 ⁻⁵ ⑤	conc ⑥	10 ⁻⁷ ⑤	conc NC
100% (+)	0	some are and cells (+)	0

about 100 i.u./ml for VEE-Ag.

PLAQUE ASSAY

- 1 ① 10⁻² ÷ 10⁻⁴
2 ② 10⁻² ÷ 10⁻⁴
3 ③ 10⁻² ÷ 10⁻⁴ 10⁻⁵ ÷ 10⁻⁷
4 ④ 10⁻² ÷ 10⁻⁴
5 ⑤ 10⁻² ÷ 10⁻⁴
6 ⑥ 10⁻² ÷ 10⁻⁴
7 ⑦ 10⁻² ÷ 10⁻⁴

large + small
4 x 10³ pfu/ml
2 x 10² large, 7 small
1 x 10⁵ pfu/ml
large

0
(2.5 x 10⁹ pfu/ml large (plus small))
0
10⁴ i.u./ml

The results suggest that LSN and
P5-1 are contaminated with VEE (+Pc
virus). However, no VEE
synthesis could be detected
in transfection efficiency
determinations (above)

2) LSN P10-1 20+2

0 0 0

A 132

LSN - positive, w/ cells in culture

③④ - practically 100% negative, but there are few "pos" cell with probab come from ① and ②

⑤ and ⑥ - negative few "positive" cell as in ③④, may come from pos ① and ②

3) P5-1 20

4) P10-1 20

a - VEE (1.2 hrs p.a.)

①	②	③
100%	100%	0 (?!)
④	⑤	⑥
0	100%	0

5) 30/4

6) NC (no RNA)

INFECTIVITY ANALYSIS OF MEDIA

(infection in CATT slides w/ 100 µl of appropriate dil; then at 39°C + 300 µl warm analysis)

a - G

10 ⁻⁵ ①	10 ⁻⁵ ②	conc ③	conc ④
2.8	9	0	0
conc ⑤	conc ⑥	10 ⁻⁴ ⑦	10 ⁻⁴ ⑧
0	0	2.5	4

2.8 x 10⁷ i.u./ml for LSN-Ag
0.9 x 10⁷ i.u./ml

a - VEE

①	②	③	④
conc some areas about 10 and only 400 for some cells	conc some areas about 10 and only 400 for some cells	conc 100% (1)	conc 100% (1)
10 ⁻⁵ ⑤	conc ⑥	10 ⁻⁷ ⑦	conc ⑧
100% (1)	0	some areas and cells (4)	0

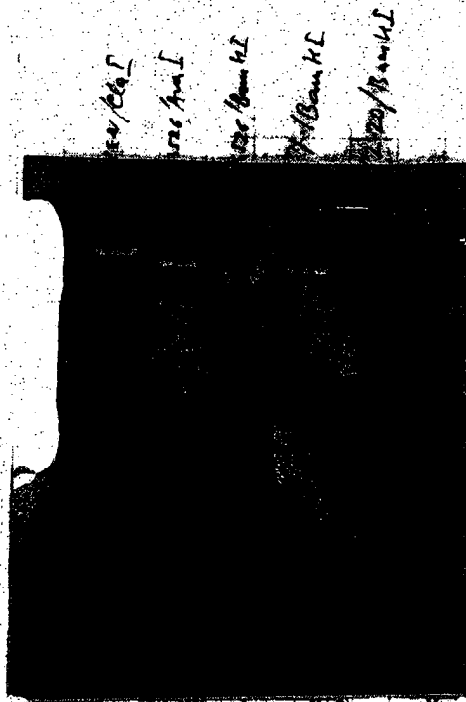
about 100 i.u./ml for VEE-Ag.

PLAQUE ASSAY

1	①	10 ⁻² = 10 ⁻⁴	large + small
2	②	10 ⁻² = 10 ⁻⁴	4 x 10 ³ pfu/ml
3	③	10 ⁻² = 10 ⁻⁴ 10 ⁻⁵ = 10 ⁻⁷	2 x 10 ² large, 7 small
4	④		1 x 10 ⁵ pfu/ml (large)
5	⑤	10 ⁻² = 10 ⁻⁴	0
6	⑥	10 ⁻⁶ = 10 ⁻⁸	(2.5 x 10 ⁹ pfu/ml large (pos small))
7	⑦	10 ⁻² = 10 ⁻⁴	0
8	⑧	10 ⁻² = 10 ⁻⁴	1 x 10 ⁴ pfu/ml large
9	⑨	10 ⁻² = 10 ⁻⁴	6 x 10 ² pfu/ml (small and large)
10	NC		0

The results suggest that LSN P5-lam contaminated with VEE virus. However, no VEE synthesis could be detected in transcription efficiency determination (above)

12/8/94

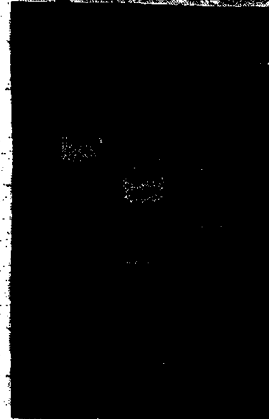


1) in both AaI - and BamHI - cleaved
3526 one additional band is present
(why?)

2) p2-20 cleavage/BamHI looks strange

12/8/94
12/8/94

No EORI site in LSN gene!



12/9/94
12/10/94
12/11/94

P5-1; P6-2; LSN were transformed into *SUSK*,
single colonies grown in 3 ml of LB-Dmp cash,
mini-preps were done with fresh LSTET & STET
(dissolved in 10 µl water - sterile)

10 µl is cleaved o/n at 37°C w/NOTI,
precautions were taken against contamination

12/12/94



2X new MneII-chloroform
+ 20 µl Na Acetate
+ 1000 µl EtOH (cold)
- 70°C

TRANSCRIPTION

	G55a	P6-2	P5-1
water	8.5	8.5	8.4
5X	8	8	8
25mM NTP/G	12	12	12
25mM cap	4.8	4.8	8.4
50A/AAI	1.5	1.5	1.5
100mM GTP	0.7	0.8	0.7
100mM			

5.26 are additional ~~can~~ present (why?)

? p2-20 cleavage/SamHI looks strange

No ECOLI site in LSN gene!

A 134

12/9/94
12/10/94
12/11/94

P5-1; P6-2; LSN were transformed into *E. coli*,
single colonies grown in 3 ml of LB-thy broth,
miniprep were done with fresh LSTET & STET
(solvent in 50 µl water - sterile)

10 µl is cleaved o/n at 37°C w/NotI,
precautions were taken against contamination

12/12/94

2x new Meust-chloroform
+ 20 µl Na Ac
+ 1000 µl EtOH (100%)
- 7000

TRANSCRIPTION

	G55a	P6-2	P5-1
water	8.5	8.5	8.4
5x	8	8	8
25mM NTP/G	12	12	12
25mM cap	4.8	4.8	8.4
500A/NotI	1.5	1.5	1.5
100mM GTP	0.7	0.8	0.7
10x B ₁₂	4	4	4

12/13/94

PLAQUE ASSAY CONTINUED

RT-PCR GibcoBRL Cat No. 18089-011 Superscript II system

RNA was extracted from cell culture medium by guanidine isothiocyanate method (John's method)

+ 13 μ l of RNA taken
 + 1 μ l of random hexamers or 1 μ l of 2 μ M/ μ l of PVDF-R2
 68°C 7 min, 1 min on ice

+ 10x Synth buffer 2 μ l
 10mM dNTP mix 1 μ l
 0.1 M DTT 2 μ l
 SS II RT 1 μ l
 7 min Room Temp; 42°C 70 min
 15 min 68°C

+ 1 μ l RNase H
 20 min at 37°C

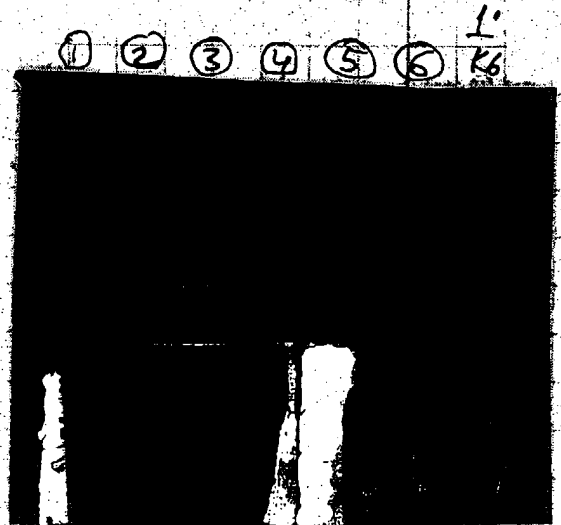
+ 1.2 μ l of VCR2
 1.8 μ l of VCF1
 8 μ l 10x synth buf
 67 μ l water
 AmpliTaq 1 μ l
 mineral oil

1' at 94°C
 and 30 cycles 1' at 94°C
 2' at 52°C
 2' at 72°C
 30 cycles

Samples:

- ① P1-5
 ② P5-1
 ③ 3014
 ④ P1-5
 ⑤ P5-1
 ⑥ 3014
- { cDNA from random hexamers
 { cDNA from E2
 { primer PVDF-R2 (Lorenz)

PCR fragments were done by C' primers
 VCF1 and VCR2 (Lorenz)
 expected size 860 bp



1/14/94

TRANSFECTION RNA, from 12/14/94 11:30

1) LSN + P6-2 13 + 18

First) time course sample was taken

9 hrs p.e.

2) LSN + P5-1 13 + 18

2nd) 21 hrs p.e.

3rd)

3) LSN

4th)

4) P5-1

IFA for determination of transfection

efficiency 21 hrs p.e.

5) P6-2

infectivity.

transf (12/14/94)

6) ~~NC~~ 100% 100%
 few positive, may be artifact
 " " " "

A

6 10⁻² 9 hrs p.e. 2 10⁻² 9
 1 10⁻⁵ 21 2 10⁻⁵ 21 hrs p.e. dilution factor
 2 10⁻⁵ 2 hrs p.e. 2 10⁻⁵ 27 hrs p.e. no of positive cells
 6 10⁻⁵ 27 hrs p.e. 63 10⁻⁵ 27 hrs p.e.

Cells about

a-Lassa:

1) LSN + P6-2
 2) LSN + P5-1
 3) LSN
 4) P5-1
 5) P6-2

VEE

80% VEE-pos
 1 conc 27 2 conc 27 3 conc 27 4 conc 27
 5 conc 27 few areas or none!
 many single cells but if only, positive areas

a-VEE

1)
 2)
 3) <10
 4) <10
 5) <10

NA analysis

by RT-PCR see next page

LA

LA

20 10⁻⁴ 2 10⁻⁴ 1 10⁻⁵ 2 10⁻⁵
 1 conc 27 2 conc 27 3 conc 27 4 conc 27
 5 conc 27 hc

Plaque assay:

1) plates 1
 2) 2
 3) 3
 4) 4

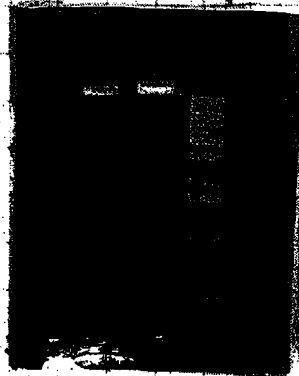
although there are few positive cells with no

thin factor
 layers 0.2
 10⁴ 10⁵ 2 dil
 each (10⁴ 10⁵)
 10⁴ 10⁵

Seawater 3526 Apr 2 Rsr II
 3014 Sma I Rsr II

12/15/94

(For synthesis)



SM

TRANSCRIPTIONS

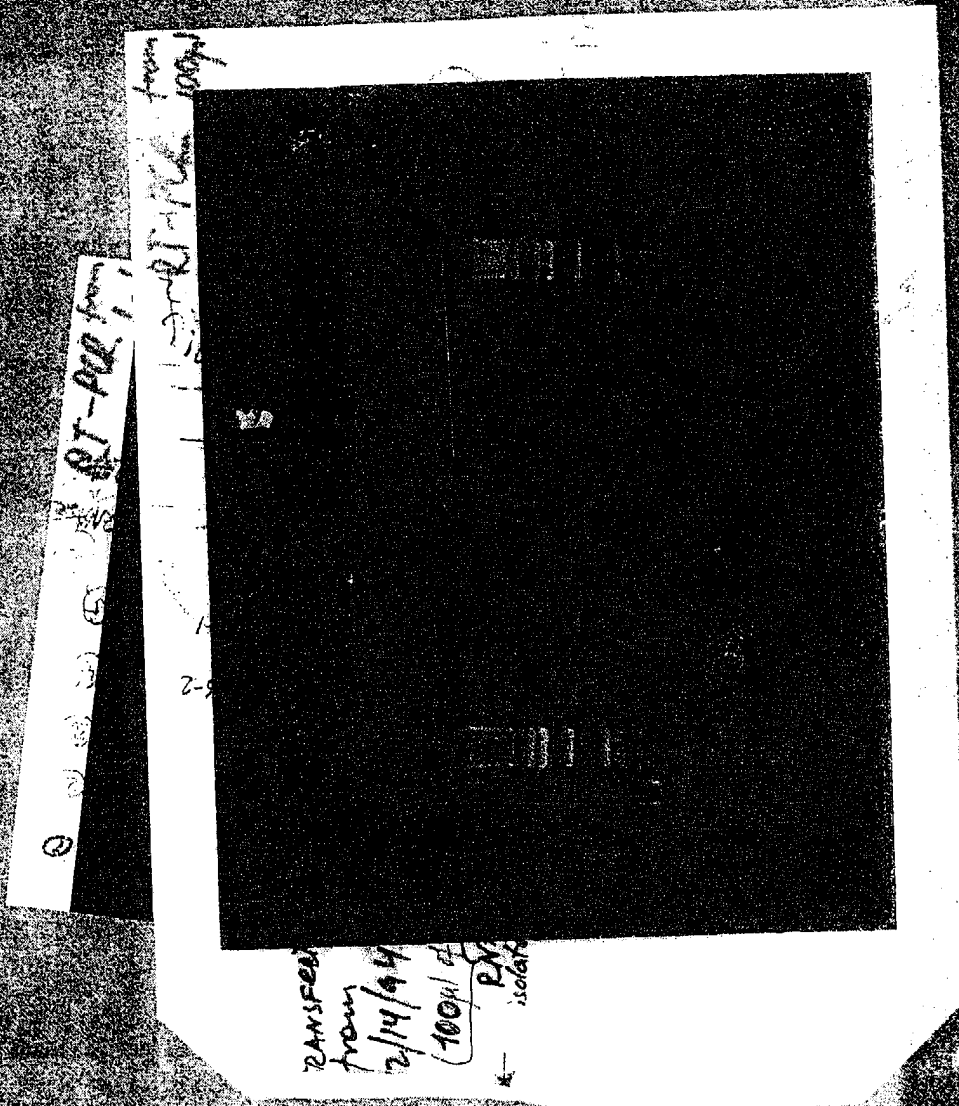
	P8-1/0 Sep	P10-1/0 Sep Rsr	LSN
water	8.8	8.8	8.5
5x	8	8	8
25 mM NTP/-G	12	12	12
25 mM cap	4.8	4.8	4.8
DNA/NOTE	1.2	1.2	1.5
100 mM GTP	0.7	0.7	0.7
10x Enz	4	4	4

4 hrs at 37°C

12/16/94

+3x 10 mg/ml RNase A
 30' at RT

Don manual (John's Method)



RT-POL from

from

TRANSFERT
from
2/14/94
(100% of
RMS
isolated

12/17/94 1) RT-PCR as in protocol 12/13/94
compare to Gibco BRL protocol

2) TFA for VEE

3)

P11 30/14 Δ RsrII SmaI 6/12 Δ + 2 μ l Lys.

P12 35/

4) TRANSFECTION

Δ RSP2	1)	(LSN) + T	12
Δ RsrSpt	2)	LSN + F	
	3)	LSN	
Δ RSP2	4)	(PB-1)	
Δ RSP2	5)	P10-1	

INFECTIVITY
(24 hr p.i.)

19 hr
p.i.

1)

1.4

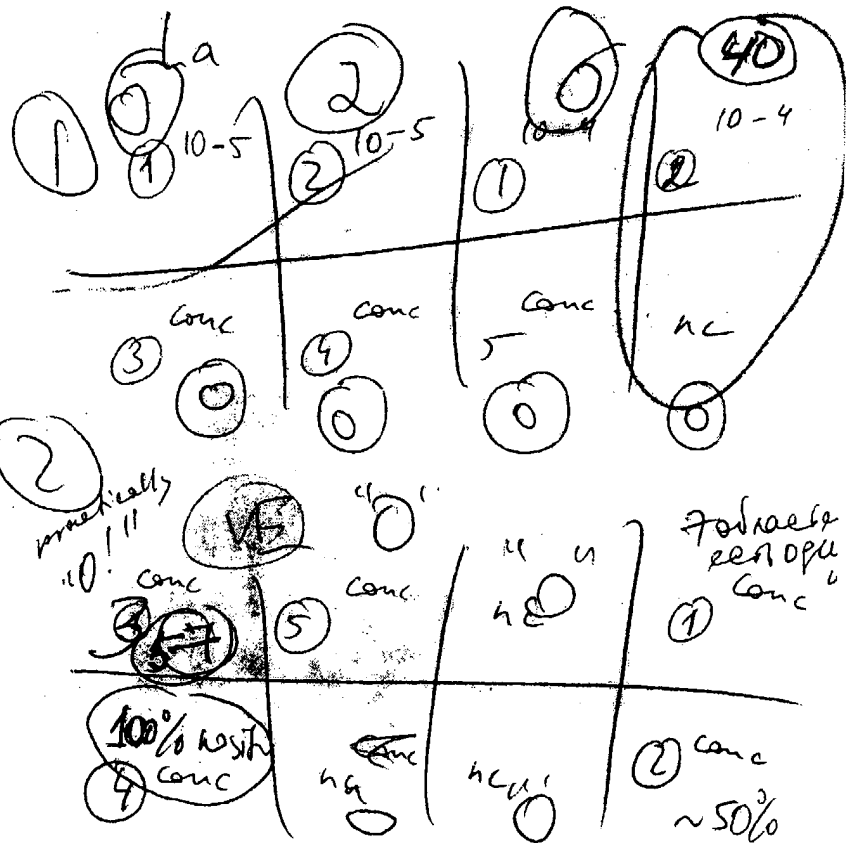
2)

3)

4)

5)

RT-PCR



3

a lot of
single cells
and parasites

only
single
cells
old

few sing
cell

many
(90%)

Plaque assay

1) Plate 6 and 1

2) Plate 2 and 1

12/18/94 1) TRANSFORMATION P11 & P12

- 2) IFA - LSN-A₂ and VEE-A₂ (transfection efficiency from 12/17/94)
- 3) RT-PCR gel (data on page 103)
- 4) INFECTIVITY ANALYSIS of transfection 12/17 & (12/14 - VEE)

A 140

12/19/94

- 1) BHK₂₁ spotted
- 2) Plaque analysis of transformations 12/14 & 12/17
- 3) IFA - infectivity analysis of 12/17 transfection - frozen at -20 after acetone
- 4) RNA extraction from 12/17 transfection
- 5) P12 - plasmids inoculated into LB-amp

- 4) 100 μ l of PBS + 1 μ l of ^{medium containing particles} sample or (pl control RNA from SScript kit)
6 - with RNase 7 without RNase

5) Ligation P11 & P12 (again)

12/20/94

- 1) Plaque assay and IFA - continue
- 2) RT-PCR
- 3) mini-preps P12 1-16, cleavage w/ SmaI
- 4) Transformation?
- 5) Transcription?
- 6) 3014 & Reverse ~~trans~~? and others?

7) TRANSCRIPTION

Water

5x

25 mM ^{ATP} GTP

25 mM ^{cap} cap

200 μ M NTPs

100 mM GTP

10x Enz

3)

P10-1

LSN

8.9

8.9

17.8

8

8

16

12

12

24

4.8

4.8

9.6

1.5

1.5

3

0.8

0.8

1.6

4

4

8

3526

TEB

all P12 1-16 are 0

P12-1

is 3526

- 2) Plaque analysis of transformations 12/14 & 12/17
- 3) IFA - infectivity analysis of 12/17 transfection - frozen at after acc
- 4) RNA extraction from 12/17 transfection
- 5) P12 - plasmids inoculated into LB-amp

4) 100 μ l of PBS + 1 μ l of ~~medium containing particles~~ sample or ~~ph~~ control RNA for SSscript
 6 - with RNase 7 without RNase

5) Ligation P11 & P12 (see V)

A 141

- 12/20/44
- 1) Plaque assay and IFA - confirm
 - 2) RT-PCR
 - 3) mini-preps P12 1-16, cleavage w/ SmaI
 - 4) Transformation?
 - 5) Transcription?
 - 6) 3014 RNAse ~~RNAse~~? and others?

7) TRANSCRIPTION

Water

5x

25 mM ~~AMP~~ ~~-G~~

25 mM ~~cap~~

25 mM ~~Na~~

100 mM ~~GTP~~

10 x ~~Enz~~

P10-1

LSN

8.9

8.9

17.8

8

8

16

12

12

24

4.8

4.8

9.6

1.5

1.5

3

0.8

0.8

1.6

4

4

8

3)

all P12 1-16 are

P12-1 is 352
 Δ ~~Age~~
 12

12/21/94 (1) TRANSFORMATIONS

- 1) P2-2 272.5 Δ μ mI
- 2) P6-3 3014 Δ μ mI
- 3) P8-2 3000 Δ μ mI
- 4) P10-2 3014 Δ μ mI
- 5) LSN - Repton

2) ~~RNA~~ RNA extraction (samples 12/17/94)

3 times, please treatment

1-8 (end ③)
 are treated
 30 min at 42
 with 50% of
 extract

not treated
 with extract

1. ① from sup's of transformations 12/17/94 1 μ l + 100 μ l PBS
2. ② — — — — —
3. ③ — — — — —
4. ④ — — — — —
5. ⑤ — — — — —
6. ⑥ 100 μ l of PBS + 0.5 μ l of 1:10 diluted control
7. ⑦ 100 μ l of PBS (- control)
8. ⑧

3) TRANSFECTIONS

1900

Efficiency (16 hrs p.e.)

		LA	VE	
1) LSN + P2-1	10 + 18	90-100%	0%	positive cells, esp. VE
2) LSN + P8-1	10 + 18	90-100%	20-30%	
3) LSN + P10-1	10 + 18	90-100%	50-60%	
4) LSN	10	90-100%	0	
5) P2-1	18	0	0	
6) P8-1	18	0	0	
7) P10-1	18	0	0	

some of the cells
 (groups) are bigger
 than the rest.
 Are these not
 transfected with
 helper?
 May be, not.

4) Plaques from P8-1 (12/17) taken (3 plaques, (+) media frozen at -70°C)

INFECTIVITY

ANALYSIS

23:30

infections (sup's take 27 hrs)

	(15 hrs p.i.)	(24 hrs p.i.)	LA (15 hrs p.i.)	24 hrs p.i.	VE (15 hrs p.i.)	
1) LSN + P2-1	<10	<10 i.u./ml	<10	<10	<10	small bright area
2) LSN + P8-1	2.5×10^4	1.7×10^4 i.u./ml	<10	<10	<10	2 large & 2 small areas, single (cells) (few)
3) LSN + P10-1	$\sim 5 \times 10^7$	nt i.u./ml	<10	<10	<10	single cells, many on edge of cells, KA
4) LSN	<10	60 i.u./ml (?)	<10	<10	<10	many 22 hr minus
5) P2-1	<10	<10 i.u./ml	<10	<10	<10	
6) P8-1	<10	<10 i.u./ml	<10	<10	<10	
7) P10-1	<10	nt	<10	<10	<10	

<10, many dead cells!

Plaque analysis

3 times plasmid treatment

2) ~~RNA~~ RNA extraction (samples 12/17/94)

- 1 = 8 (ex 7) 1. ① four sup's of transfection 12/17/94 1 μ l + 100 μ l PBS
 ate treated 2. ② — — — — —
 30 min at 45 3. ③ — — — — —
 with 50% of 4. ④ — — — — —
 with LSN 5. ⑤ — — — — —
 not treated 6. ⑥ 100 μ l of PBS + 0.5 μ l of 1:10 diluted control RNA
 with LSN 7. ⑦ — — — — —
 8. ⑧ 100 μ l of PBS (- control)

3) TRANSFECTIONS 1900 Efficiency (16 hrs p.e.)

1) LSN + P2-1	10 + 18	90-100%	Same of the cells (groups) are brighter than the rest Are these not transfected with helper? May be, not.	0%	page two well explain see very
2) LSN + P8-1	10 + 18	90-100%		20-30%	
3) LSN + P10-1	10 + 18	90-100%		50-60%	
4) LSN	10	90-100%		0	
5) P2-1	18	0	0		
6) P8-1	18	0	0		
7) P10-1	18	0	0		

4) Plaques from P8-1 (12/17) taken (3 plaques, (+) medium frozen at -70°C)

INFECTIVITY ANALYSIS 23:30 infection (sup's taken 27 hrs p.e.)

	(15 hrs p.i.)	(24 hrs p.i.)	(15 hrs p.i.)	(24 hrs p.i.)	VE (15 hrs p.i.)
1) LSN + P2-1	< 10	< 10 i.u./ml	< 10	< 10	small bright area
2) LSN + P8-1	2.5 x 10 ⁴	1.7 x 10 ⁴ i.u./ml	< 10	< 10	2 large & 2 small areas (20% simple cells, few)
3) LSN + P10-1	~ 5 x 10 ⁷	nt i.u./ml	< 10	< 10	single cells, many p.p. present, known
4) LSN	< 10	60 i.u./ml (!?)	< 10	< 10/ml	has 2200 neurites
5) P2-1	< 10	< 10 i.u./ml	< 10	< 10/ml	—
6) P8-1	< 10; many dead cells	< 10 i.u./ml	< 10	< 10/ml	—
7) P10-1	< 10	nt	< 10	< 10/ml	—

Plaque analysis

- 1) undiluted (10⁻¹) = 10⁻³
 2) undiluted (10⁻¹) = 10⁻³
 3) undiluted (10⁻¹) = 10⁻³
 4) undiluted (10⁻¹) = 10⁻³

7) 10⁻¹ undiluted (10⁻¹)

94 jmk Setup Listing

Version 2

15:08P 12/22/94

394 Atresis: Setup Listing

SINGLE X 01 Abs Read average = 01

SH 260.0

SH 260.0

1 0.0059
 2 PO1 0.6316 1,042.14 $\mu\text{g/ml}$ (1 mg/ml) 179.6 μM
 3 PO2 0.6667 1,100.06 $\mu\text{g/ml}$ (1.1 mg/ml) 201.9 μM

S- 0.6- 0.7- 0.8- 0

000000 0)

TV: 5567.6

OD x dil x 33 = $\mu\text{g/ml}$

S x BTS TAC AAC GAA CBT GAG x3

at 1423

at 1940

 $\text{mg/ml} / \text{MW} = (\text{M})$

12/23/94

- ① Gel RT-PCR - for structural ϵ^{ca} gene (12/17 samples) data on p.
- ② Intensity analysis - IFA a-LSN & a-VER
- ③ PCR primers; PCR for usp genes ???
- ④ Minipreps (5 or 6); cleavage w/ NotI
- ⑤ Infection (12/21 samples) 19:00

12/24/94

- 1) IFAs (acetone $\rightarrow -20^{\circ}\text{C}$) 15 & 24 hrs p.i.
- 2) NotI cleavage - check(!), purify, precipitate $\rightarrow -70^{\circ}\text{C}$

12/29/94



TRANSCRIPTIONS

LSN/NotI old

P2-2/Not

P8-2/Not

P12-1/Not

} new

12/26/94 TRANSFECTION 19:00 Efficiency (20 hrs p.e.)

gives plaques
3 days after starting w/ neutral test

			CA	VE
1.	LSN + P12-1	10 + 19	90% - 100%	10-25%
2.	LSN + P2-2	10 + 19	90% - 100%	0!!
3.	LSN + P8-2	10 + 19	90%	5-10%
4.	LSN	10	90%	0
5.	P12-1	19	0	0
6.	P2-2	19	0	0
7.	P8-2	19	0	0

RC2: Note template contains only 400 bp fragment

Media were taken (1ml of Nos 1-7) 28 hrs p.e., incubation continued at 37°C, and samples were taken

INFECTION OF BHK cells (inlet Ten slides) 19:00 (19 hrs p.i.)

with media collected either 28 hrs p.e. or 47 hrs p.e.

	28 hrs p.e.	47 hrs p.e.	28 hrs p.e.	47 hrs p.e.
1 LSN + P12-1	$< 10^3$	$< 10^3$ i.u./ml	10 ⁴ i.u./ml	80%
2 LSN + P2-2	< 10	< 10	< 10	< 10
3 LSN + P8-2	340 i.u./ml	10 ⁴ i.u./ml, 30% dead cells	60%	100%
4 LSN	< 10	< 10	10 ⁴ i.u./ml	< 10
5 P12-1	< 10	< 10	< 10	< 10
6 P2-2	< 10	< 10	< 10	< 10
7 P8-2	< 10	< 10	< 10	< 10

Some possible single cells found many possible single cells (dead)

Plaque assay see on p. 111

Helpers with KpnI fragment deleted (ns2 region)

Construct	Transfection date	Transfection efficiency, by IFA, %		Hrs p.e.	Media collected, hrs. p.e.	INFECTIVITY by IFA		Hrs. p.i.	PLAQUE ASSAY
		anti-L	anti-V			anti-L, i.u./ml	anti-V, i.u./ml & f.f.u./ml		
LSN	12/26/94	90	0	20	28 47	<10 <10	<10 <10	19	
LSN+P2-2		90-100	0		28 & 47	<10 & <10	<10 & <10		
P2-2		0	0		28 & 47	<10 & <10	<10 & <10		
LSN+P8-2		90	5-10		28 47	340 singles 10 ⁴ (few areas 10 cells each + singles)	60% 100%		
P8-2		0	0		28 & 47	<10 & <10	<10 & <10		
LSN	12/21/94	90-100	0	16	27	<10	<10		
LSN+P8-1		90-100	20-30			1.7x10 ⁴ 2.5x10 ⁴	30ffu/ml 100ffu/ml 100iu/ml	15 24	
P8-1		0	0			<10 <10, many dead cells in undiluted smpl	<10 <10	15 24	
LSN+P2-1		90-100	0			<10	<10 or 10 ffu/ml		
P2-1		0	0			<10	<10		
LSN	12/17/94	100	0	15	24	<10	<10		<10
LSN+P8-1		100	5-25			1.4x10 ⁴	70ffu/ml+ single cells	19	7x10 ²
P8-1		0	0			<10	100%		3x10 ⁵
LSN	12/14/94	100	0	21	27	<10	<10		<10
LSN+P6-2		100	100			6x10 ⁶	80-100%	19	10 ⁵
P6-2		0	0			<10	<10		<10
LSN+P6-2	11/30/94	100	70-80	22	27	4.5x10 ⁶	2000ffu/ml	27	7x10 ²
P6-2		0	0			<10	100%(undil)		7x10 ⁶
LSN+P6-2	CEF 11/26/94	5	0	?	?	?	80%(undil)		0 - 10 ²
P6-2		0	0	?	?	?	nt		0
LSN+P2-1	10/24/94	60-70	<1	22	?	?	?		0
LSN+P2-1	10/13/94	?	?	?	?	<10 ³	<10 ³		?
LSN	10/1/94	60-80	0	18	?	nt	nt		nt
LSN+P2-1		60-80	0			<10 ⁴	<10 ⁴		nt
P2-1		0	0			nt	nt		nt

ΔKpn I helpers

Donor	Date of transfection	Efficiency of transfection	p.e., hrs	Media collected, hrs	LA	IFA, T ₄ /I	Plaque forming units
LA	UE						
SN		90%					
SN+2-2	12/26	90%	20	28 and 47			
SN+8-2		90%					
2-2		0					
8-2		0					
CSN	12/21/94	90-100%	16	27	<10	<10	
+P8-1	12/21/94	90-100%	16	27	1.7×10^4 /15 hrs p.i.	30 areas/m ²	
					2.5×10^4 /24 hrs p.i.	160 areas/m ²	
						+ single cells	
P8-1	12/21/94		16	27	<10/15 hrs p.i.	<10/24 hrs p.i.	
					<10, many dead cells	<10/24 hrs p.i.	
CSN	12/17/94	100%	15	24	<10	<10	
+P8-1	12/17/94	100%	15	24	1.4×10^4 /19 hrs p.i.	70 areas/m ²	
						+ single cells	
P8-1	12/17/94		15	24	<10	100% of cells are positive	
CSN	12/14/94	100%	21	27	<10	<10	
P6-2	12/14/94	100%	21	27	6×10^6 /27 hrs	80-100% positive/19 hrs	
P6-2	12/14/94		21	27	<10	<10	
+P6-2	11/30/94	100%	22	27	4.5×10^6 /27 hrs	2×10^3 areas/m ²	
P6-2	11/30/94		22	27	<10	all positive (and more)	
+P6-2	11/26/94	5%	?	?	?	80% positive	
P6-2	CHICK	0	?	?	?	nt	
CSN	12/21/94	90%-100%	16	27	<10	<10	
+P2-1	- - -	90-100%	16	27	<10	<10	
P2-1	- - -	0	16	27	<10	<10	
+P2-1	10/24/94	60-70%	22				
+P2-1	10/13/94	?	?				
CSN	10/1/94	60-80%	18				
CSN+P2-1		60-80%	18				
P2-1		0	18				

6/1/95

DNA PCR

- (1) LSN template new (48 primers) 100°C annealing 0.1 M DNA
 (2) P8-2 template — (— 10 M — — —
 (3) LSN template — (— 200 M — — —
 (4) old P8-2 (PCR grade) — (— 10 M — — 0.5 M DNA
 (5) — 11 same samples annealing
 (6) — 2
 (7) — 3
 (8) — 4



1
2
3
4

TRANSFECTION

12:00

4/8/95

p.e.
samples

1. LSN + P2-2
2. P2-2
3. LSN + P6-3
4. P6-3
5. LSN + P8-2
6. P8-2
7. cells electroporated
8. cells not electroporated

18:00

IFA w/samples collected
(undiluted)

1. LSN + P2-2
2. P2-2
3. LSN + P6-3
4. P6-3
5. LSN + P8-2
6. P8-2

2 hrs p.e.

50 hrs

<10

<10

Recombinants
just bright

<10

<10

<10

 $\sim 1 \times 10^5 / \text{ml}$

100% chaos E?

100%

100%

<10

<10

<10

 $\sim 6 \times 10^3 / \text{ml}$ ~~5-6~~ $\sim 5-6 \times 10^3 / \text{ml}$

100%

100%

<10

<10

<10

($\leq$) means there w
mostly cells on
plate. If there
are more than
the
(10⁵)
from this

18:00

Plaque assay

 $n^2 = 5 \text{ pfu/ml}$ (undiluted Sup-8 + diluent & 100 μl per well)

Samples 27 & 50 hrs p.e.

1. (1) 27 hrs p.e. $10^{-2} = 10^{-4}$ < 100
2. (2) 27 hrs p.e. < 100
3. (3) 27 hrs p.e. $5 \times 10^3 / (0.2-3 \text{ mm})$
4. (4) 27 hrs p.e. < 100
5. (5) 27 hrs p.e. $1 \times 10^3 / (0.2-1 \text{ mm})$
6. (6) 27 hrs p.e. < 100
7. (7) 50 hrs p.e. < 100
8. (8) 50 hrs p.e. < 100
9. (9) 50 hrs p.e. $\sim 1.5 \times 10^6 / (0.2-3 \text{ mm})$

13. (1) LSN P2-1 < 100
14. (2) LSN P2-2 < 100
15. (3) LSN P8-2 $6 \times 10^3 / (0.2-1 \text{ mm})$
16. (4) P2-1 < 100
17. (5) P2-2 < 100
18. (6) P8-2 < 100

1×10^5 65 hrs
(0.2-1 mm)

27 hrs p.e.; $10^{-2} = 10^{-4}$ dil

TRANSFECTION 10/2

-

May be, reasoner
already exist

many cells

2. (H4) 101

50 hrs

Recombinants
list are

- mean
there
not

probably had cells
in the dark area
which were from
the same place as
the ones from
sample or from #

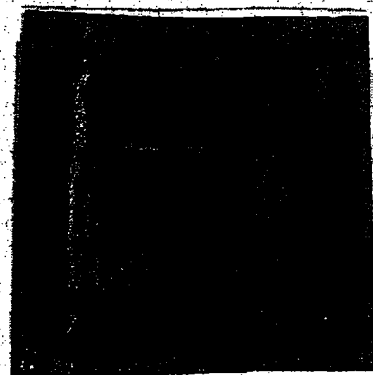
(undiluted sup-3 + diluent 2 100 μ l per well)

$$n^{-2} \div n^{-4} = n^2$$

Samples 27 & 50 hrs p.e.

- ## TRANSFECTION

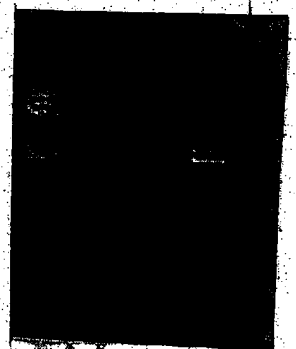
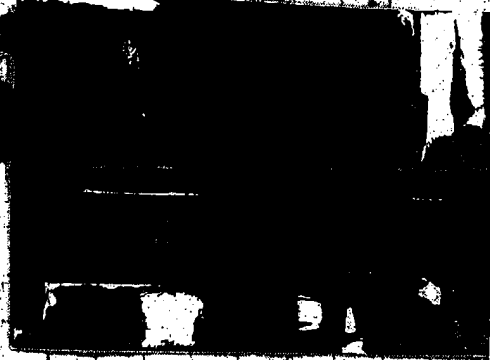
Cleavage 3014 1) Aat II
2) Kpn I



P13	3014 Δ Aat II	5 μl + 10 μl water + 2 μl C + 2 μl G
P14	3014 Δ Aat II T4	5 μl + 11 μl water +
P15	3014 Δ Kpn I T4	5 μl + 11 μl water +
P16	3014 Δ Kpn I T4	1 μl + 15 μl water +

1/13/95

1/13/95



Further cleavage

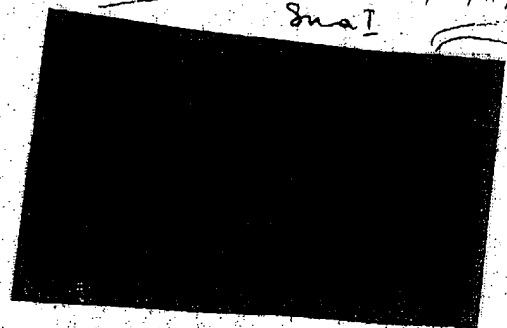
P13 5
P14 5

P15 7; 8; 9; 11; 13

3014 P13 1-5 P14 1-4 P15 1-5 3014



P15 7, 8, 9, 11, 13
8 μl
3014



Cleaveage

P13 1,2 } Aat II Rsr II
 P14 2,3 }
 P15 7,8,9 } Kpn I Rsr II

Aat II Rsr II
 P13 Aat II Rsr II
 P14 Aat II Rsr II
 P15 Kpn I Rsr II

P13-1: 3014 Δ Aat IIP13-2: 3014 Δ Aat IIP14-3: 3014 Δ Aat II / T4 posP15-7: 3014 Δ Kpn I / T4 posP15-8: 3014 Δ Kpn I / T4 posP15-9: 3014 Δ Kpn I / T4 pos

P13-2 }
 P14-3 } Not I o/n ; Kpn I
 P15-7 } Aat II
 Kpn I Kpn I

1/19/95

TRANSCRIPTIONS

LSN

P13-2

P14-3

P15-7

TRANSCRIPTION
12/28/94

Results of PLaque Assay (24 hrs post stain) 12/28/95

1. ① - 28 hrs	LSN-1 < 10 ²	1 x 10 ³ (65 hrs post staining)	0.2 - 1 mm ϕ	5 x 10 ³
2. ① - 47 hrs	P12-1 < 10 ²	4 x 10 ⁴ (0.2) 1 mm		5 x 10 ⁴
3. ⑤ - 28 hrs	< 10 ²	< 10 ²		< 10 ²
4. ⑤ - 77 hrs	P12-1 < 10 ²	< 10 ²		< 10 ²
5. ① LSN + P2-1	< 10 ²	< 10 ²		< 10 ²
6. ② LSN + P8-1	< 10 ²	1 x 10 ³ 0.1 - 0.2 mm		5 x 10 ³

P 14-3 : 3014 Δ Aat I / T4 pos

P 15-7 : 3014 Δ Kpn I / T4 pos
 P 15-8 : 3014 Δ Kpn I / T4 pos
 P 15-9 : 3014 Δ Kpn I / T4 pos

A 153

P 13-2 { Not I o/n } Kpa I
 P 14-3 { Aat I
 P 15-7 { Kpa I Kpn I

1/19/95

TRANSCRIPTIONS
 CSN
 P13-2
 P14-3
 P15-2

TRANSFECTION
 12/26/94

Results of PLaque assay (24 hrs post stain)

TRANSFECTION
 12/21/94

1. ① - 28 hrs	CSN < 10 ²	1 x 10 ³ (65 hrs post staining)	0.2 - 1 mm ϕ	5 x 10
2. ① - 47 hrs	P12-1 < 10 ²	4 x 10 ⁴ (0.2) ϕ mm		5 x
3. ⑤ - 28 hrs	< 10 ²	< 10 ²		< 10
4. ⑤ - 47 hrs	P12-1 < 10 ²	< 10 ²		< 10
5. ①	CSN + P2-1 < 10 ²	< 10 ²		< 10
6. ②	CSN + P8-1 < 10 ²	1 x 10 ³	0.1 - 0.2 mm	5 x 10
7. ③	CSN + P10-1	2 x 10 ² (0.1 - 1 mm)	+ 0.2 - 1 mm ave (6 hrs) = ~ 1 x 10 ³ total (65 hrs)	1 x 10
8. ⑤	P2-1 < 10 ²	< 10 ²		< 10
9. ⑥	P3-1 < 10 ²	< 10 ²		< 10
10. ⑦	P10-1 < 10 ²	< 10 ²		< 10

4/21/94 TRANSFECTION

11am

1) LSN + P13-2
2) LSN + P14-3
3) LSN + P15-7
4) P13-2
5) P14-3
6) P15-7

a-LA

90-100%

90-100%

90-100%

0%

0%

0%

0%

22hrs p.e.

a-VE

80-90%

80-90%

40-50%

0%

0%

0%

22hrs p.e.

looks l
ahead
and i
cells.

22hrs p.e.

22hrs p.e.

Media Samples were taken

29hrs p.e.

54hrs p.e.

72hrs p.e.

LSN
29 54 72
1
2 } used circular (10⁻⁴) papers
3
4 ut
5 nt
6 nt

90-100%
90-100%
90-100%
90-100%
90-100%
90-100%

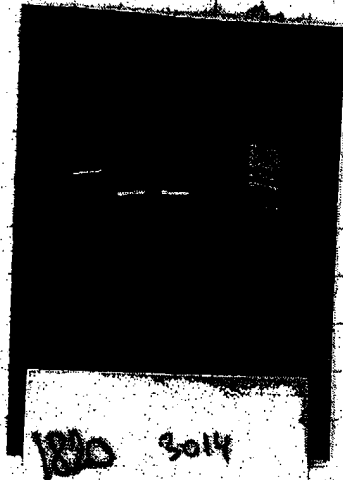
Diapase assay

10⁻² = 10⁻⁴

1. 8/1-① - 27hr LSN+P2-2 <100
2. 8/1-① - 50hr - - - <100
3. 8/1-① - 78hr - - - <100
4. 8/1-③ - 27 LSN+P6-3 4x10³ (0.1-1.5) up to 1x10⁴
5. - - - 50 - - - 4x10³ (0.1-3) + smalls
6. - - - 78 - - - 8x10³ (0.1-2) + smalls
7. 8/1-⑤ - 27 LSN+P8-2 1x10³ (0.1) 1x10³ (+5?)
8. - - - 50 - - - 5x10⁶ (0.1-1) + smalls
9. - - - 78 - - - 5x10⁶ + smalls
10. 8/1-④ - 27 P6-3 <100
11. 8/1-④ - 50 P6-3 <100
12. - - - 78 P6-3 <100
13. 12/14/94 LSN P5-1
14. 21/1-① - 29hr LSN+P13-2 <100 in 10⁻⁴ and 2 spots
15. - - - ② - - - LSN+P14-3 1x10³ (1mm)
16. - - - ③ - - - LSN+P15-2 1x10³ (0.5-1)
17. - - - ④ - - - P13-2 3x10⁶
18. - - - ⑤ - - - P14-3 ~1x10⁴
19. - - - ⑥ - - - P15-2 ~1x10⁴
20. 12/14/94 LSN P5-1 undil (10⁻¹) <10 of cl

1/24/95

PCR of (1) 3014 (2) P13-2 (3) P14-3 (4) P15-7 [all Not-cleaned
primers P01
P02
transcription
templates]



180 3014

100% transcribed
40% success

Purification of RNA from

(5) 1/21/95 (P14-3, 10^7 pfu/ml)

medium cleared 10' at 4800 rpm

- 1) 600 μ l of medium + 600 μ l phenol-chloroform
- 2) 600 μ l of medium + 10 μ l RNase
- 3) 600 μ l of medium + 10 μ l RNase } 30' at RT + 600 μ l phenol-chloro

2.5 vol. EtOH (RNA grade) 0.5 vol NaAc
2% Glycogen solution (Genius kit)

1/25/95

Purification of RNA

1	12/14/94	P5-1 + 15 μ l	250 μ l H ₂ O	
2			+ 10 μ l RNase	30' at RT
3	P14-3	1/21/95	250 + 250	+ 10 μ l RNase
4			250 + 250	+ 10 μ l RNase + 5 μ l DNAse
5	P15-7	1/21/95	250 + 250	
6			250 + 250	+ 10 μ l RNase
7	medium		250 + 250	+ 10 μ l RNase
8	medium		250 + 250	+ 10 μ l RNase + 5 μ l DNAse

RNA isolation from growing media

1.	14-3	250 + 250	+ (10) μ l RNAse	
2.	14-3	250 + 250	+ (10) — — —	+ (5) μ l DNase
3.	medium	250 + 250	+ (10) — — —	
4.	— — —	— — —	+ (10) — — —	+ (5)
5.	water	500	+ (10)	
6.	water	500	—	no phenol
7.	P15-7	250 + 250	no RNAse no DNase	

phenol

phenol

1 μ l of Glycogen+ 200 μ l
waterGlycogen or
ultrapure

- 1) infect ④ LABTEK - Burk cells with P5-1 & LSN particles
dilutions 10^{-1} ; 10^{-2} ; 10^{-4} ; 10^{-5}

1/27/94

- 1) tora-VEE ab
- 2) tora-LSN con
- 3) day 21 mouse ab (4) + NC
- 4) day 35 mouse ab + NC

infect + 75 with P5-1 & LSN particles

2) Plaque assay

1. P5-1 $10^{-2} \div 10^{-7}$

2. P5-1 $10^{-2} \div 10^{-7}$

3. P5-1 $10^{-2} \div 10^{-7}$

4. P5-1 $10^{-3} \div 10^{-7}$

5. P5-1 $10^{-3} \div 10^{-8}$

6. P5-1 $10^{-3} \div 10^{-8}$

7. P5-1 $10^{-3} \div 10^{-8}$

8. 12/26 ② 28 $10^{-2} \div 10^{-7}$

9. 12/26 ③ 47 $10^{-2} \div 10^{-7}$

10. 12/26 LSN + P5-2 47 hr $10^{-2} \div 10^{-7}$

11. 12/26 P5-1 $10^{-2} \div 10^{-7}$

12. $10^{-2} \div 10^{-7}$

on Sunday, next day
after starting
 $10^3 \div 1 \times 10^4$? very small

3×10^3

$\sim 1 \times 10^7 - 10^8$

$10^2 \div 10^{-7}$

$10^{-2} \div 10^{-7}$

$10^{-2} \div 10^{-7}$

6×10^4

$\sim 1 \times 10^7 - 10^8$

$\sim 1 \times 10^7 - 10^8$

⑤ hrs p.i.

0.5×10^3 L & 4x

X

1×10^3 L & 5x10

1×10^3 L & 5x10

2×10^3 L & 5x10

1×10^3 L & 5x10

1×10^3 L & 5x10

5×10^3

1/1995

TRANSFECTION

		(12) hr		unlabelled	labelled	labelled	by IFA efficiency of transfection		
		monolayer	CPE*	medium	cells	medium	a-RA	16 hr p.e.	a-VE
5 min marks	** 1.	P5-1 & LSN	~50-60%	NO	+	-	0	100%	50-70%
	** 2.	P5-1 & HA	~50-60%	NO	+	-	100	0%	50-70%
	* 3.	P12-3 & LSN	~50-60%	NO	+	-	0	100%	50-70%
	4.	LSN	~50-60%	NO	-	-	0	100%	0
	5.	HA	~50-60%	NO	-	-	100	0%	0
times 385 x	* 6.	P5-1	~50-60%	NO	-	-	0	0%	0
	* 7.	P12-3	~50-60%	NO	-	-	0	0%	0
	8.	-	~50-60%	NO	-	-	0	0	0
		(20 min 5 min max)					floating cells		
		monolayer	CPE*	unlabelled medium	labelled cells	labelled medium			
	1.	~50-60%	no/begin?	+	+	+			
	2.	~50-60%	no/begin?	+	+	+			
	3.	~50-60%	no/begin?	+	+	+			
	4.	same as above	-	-	-	-			
	5.	same	-	-	-	-			
	6.	same	-	-	-	-			
	7.	same	-	-	-	-			
	8.	same	-	-	-	-			
		(33) hr							
		monolayer	CPE*	unlabelled medium	labelled cells	labelled med			
	1.	~40-50%	begin	+	+	+			
	2.	~40-50	begin	+	+	+			
	3.	~40-50	begin	+	+	+			
	4.	~50	no/begin	+	+	+			
	5.	~50	no/begin	+	+	+			
	6.	~50-60	no	+	+	+			
	7.	~50-60	no	+	+	+			
	8.	~50-60	no	+	+	+			

~~P20~~: 3014: Δ Aat II Apa I

P19-1: P5-1 Δ T^HIII SpeI (Δ capsid)



4/4/95 P21: P5-1 Δ Hpa I

2NA	1 μ l
10x B1	2
water	15
Lig	2

6/6/95 P22: P5-1 Δ Hpa I - Spe I - T4 pol

P23: 3014 Δ Apa I - Aat II - T4 pol

3/7/95 TRANSCRIPTION HA and LSN

water	8.5	8.5
5x bgt	8	8
25MM ATP-G	12	12
25MM cap	4.8	~~4.8~~ 4.8
2NA/Not	2	2
100MM GTP	0.7	~~0.8~~ 0.9
10x S ₁₂	4	4

HA	LA	5-1	21-1	22-1	19-1
~~30~~	~~30~~	~~30~~	~~30~~	~~30~~	~~30~~
~~30~~	~~30~~	~~30~~	~~30~~	~~30~~	~~30~~
~~30~~	~~30~~	~~30~~	~~30~~	~~30~~	~~30~~
19	56	19	28	28	38
of mix	mix	mix	mix	mix	mix
1.5	4.5	2	4	4	5

8
12
(12) 4.8
100 mm
0.8

water
5 x 6 μ
25 mM NTP/-G
25 mM cap
DNA/Not I
100 mM GTP
10 x Σ enz

20VAs/Not I:

RNA5 (1.5 μ g each
after 4 hr
at 37°C)

24/95

TRANSCRIPTIONS

water
5x
25 mM NTP/-G
25 mM cap
DWA/Not L
100 mM GPD
60 x 5 mm

LSN	HA	P5-1	P22-1	P19-1
7.4	7.4	2.9	1.8	1.7
8	8	8	8	8
12	12	12	12	12
4.8	4.8	8.4	9.6	9.6
3	3	4	4	4
0.8	0.8	0.7	0.6(0.5)	0.7
4	4	4	4	4

1:1.5 1:3 1:4 1:3.4
GTP: cap (1:4.8)

4/4/95 P23: P5-1: Spe I T4 att_n nucleocapsid → frame shift
no GPs ORF

4/6/95 In vitro transcriptions

	HA	LSN	P5-1	P21-1
water	3.5	7.4	2.9	0.9
5x bux	4	8	8	4
25 mM NTP/-G	6	12	12	6
25 mM cap	2.4	4.8	8.4	4.8
RNA/Not	2	4	4	2
100 mM GTP	0.4	0.8	0.7	0.25
10x 8m	2	4	4	2
	20 μ l	40 μ l	40 μ l	20 μ l

4/9/95

	LSN	HA	S-1
water	9	9.0 (9.0)	5
5x	8	8 (8)	8
25 mM NTP/-G	12	12 (12)	12
25 mM cap	4.8	4.8 (4.6)	8.4
RNA/Not	1.5	1.5 (1.7)	2
100 mM GTP	0.8	0.8 (0.7)	0.7
10x 8m	4	4 (4)	4

4/10/95

	P23-2	P21-1
water	5	2.8
5x	8	8
25 mM NTP/-G	12	12
25 mM cap	8.4	9.6
RNA/Not	2 μ l	3 μ l
100 mM GTP	0.7	0.6
10x 8m	4	4

— LSN counts

4/21/95 Oligonucleotides for GPs helper

$$CA1 \quad 0.14 \text{ o.u.} \times 100 \times 33 \text{ } \mu\text{g/ml} = 462 \text{ } \mu\text{g/ml}$$

$$CA2 \quad 0.27 \text{ o.u.} \times 100 \times 33 \text{ } \mu\text{g/ml} = 891 \text{ } \mu\text{g/ml}$$

$$CA1; \quad 1 \text{ } \mu\text{l} + 46.2 \text{ } \mu\text{l} \text{ water} = 10.0 \text{ } \mu\text{g/ml}$$

$$CA2; \quad 1 \text{ } \mu\text{l} + 89.1 \text{ } \mu\text{l} \text{ water} = 10.0 \text{ } \mu\text{g/ml}$$

Annealing mixture

10 μl CA1; 10 $\mu\text{g/ml}$

10 μl CA2; 10 $\mu\text{g/ml}$

2.5 μl 5x Sequenase buffer

24.5 μl ~~water~~ 15 min

anneal to 25°C slowly

85°C

42°C 15 min

37°C 15 min

Room temperature 1 hr

P24

vector 1 μl

fragment 1 μl

10x Lig Buf 2

Ligase 2

Water 14

P24-1

(+) vector 1

10x 2

Lig 2

Water 15

(1+) Vector 1

fragm 1

10x 2

Lig 2

Water 14

(1+) vector 1

fragm 5

10x 2

Lig 2

Water 10

P24 has annealed oligos CA1 and CA2 instead
TthIII-E-Spe of P5-1

15 P24: colonies from ligation
which contained cleaved TthIII-E + Spe vector (not)
purified from gel) and 1:10 dilution of
annealing mixture 4/21

A 163

from sixteen colonies 6 were positive

purified with
Wizard PCR prep kit

26/95 Transfection

BHK-80

		L	V-Rab	V-Moy
1)	LSN + (6-2)			
2.	LSN + (10-1)			
3.	LSN + (16-1)	1 90%	10-20	10-20
4.	LSN + (23-1)	2 90%	10-20	10-20
5.	LSN	3 60%	0-20	10-20
6.	(6-2) and (10-1)	4 90%	10-20	10-20
7.	(16-1) and (23-1)	5 50% (acc. NA) 0	0	1 +/- 2
8.		6 0%	0	0
9.		7 0%	0	0

3acervum

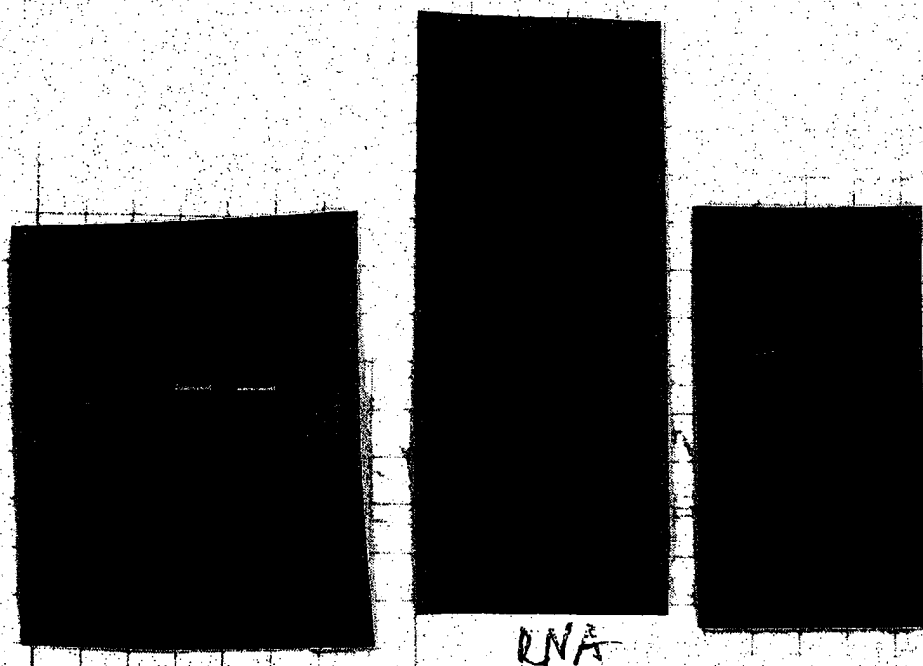
		22 hrs p.e.
①	LSN - for transfection	5
②	H4	1
③	P5-1	2
④	23-2 (5-1 Spe T4) - for	7
5.	24-3 (5-1 + acc) for	2

recovery

4/28/45

TRANSCRIPTIONS

	LSN	HA	P5-1	P24-3	P21-1	P19
water	~~10.2~~ 7	4.2	2.0	1.5	1.5	2.0
5x	8	4	4	4	4	4
25mM NTP/g	12	5	6	6	6	6
25mM cap	9.8	2.4	4.2	4.2	4.8	4.2
DNA/Not I	1.1	1	1.5	2	1.5	1.5
100mM LTP	0.8	0.4	0.35	0.35	0.25	0.3
10x Eng.	2	2	2	2	2	2



RNA

5 TRANSFECTIONS

1. ~~LSN + P5-1~~ ~~PS-1~~
 2. ~~LSN + P5-1~~

1. LSN + P5-1

1:1000 and 1:10⁻⁵2. ~~HA + P5-1~~

3. LSN + P24-3 GPC

conc

4. LSN + P21-1 C

conc

5. LSN + P19-1 GPC

conc

6. LSN + P24-3 + P21-1 C+GPC conc and 1:1, 1:10

7. LSN

conc

8. P5-1 + P24-3 + ~~HA~~

conc

9. P21-1 + P19-1

conc

TRANSCRIPTIONS

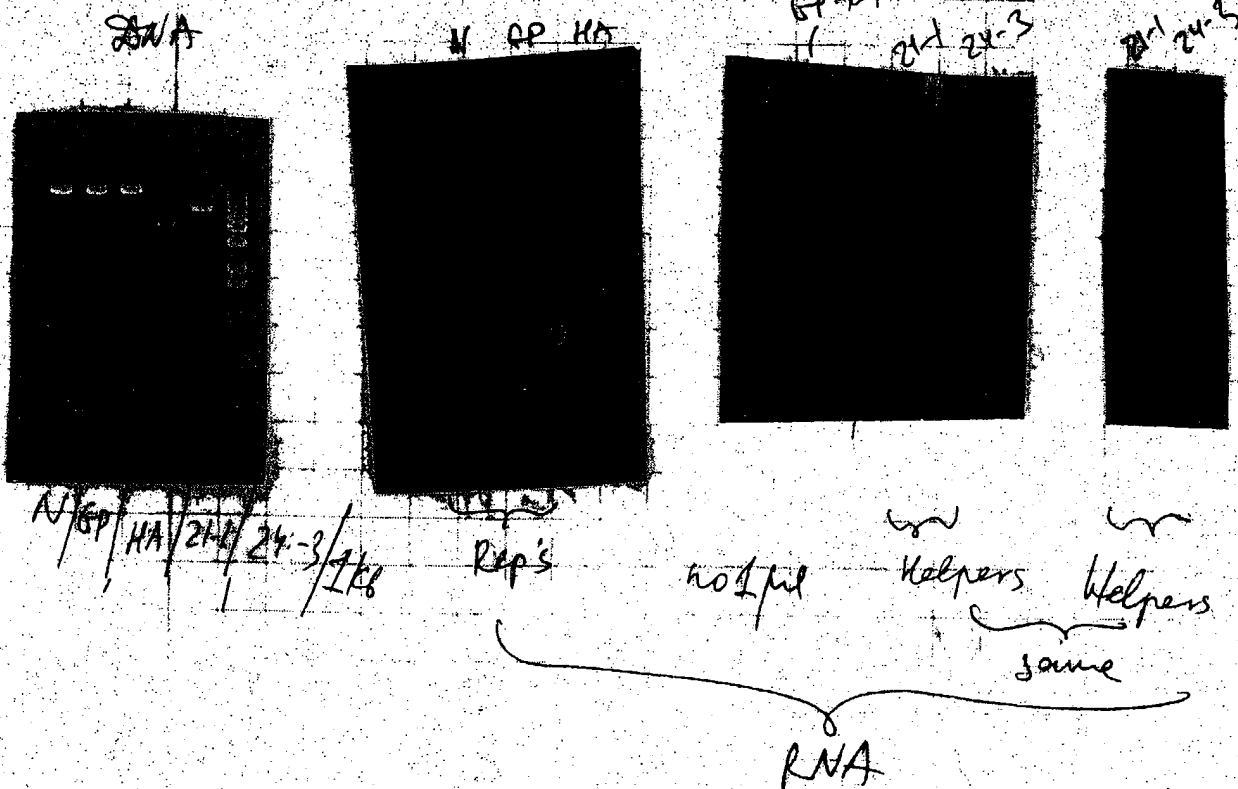
	N-Rep	GP-Rep	HA-Rep	21-1	24-3
Water	4.9	4.7	4.2	4.9	5.9
5x	4	4	4	8	8
25MM HP/G	6	6	6	12	12
25MM cap	2.4	2.4	2.4	9.6	8.4
SWA/Not I	0.3	0.5	1	1	1
100MM GTP	0.4	0.4	0.4	0.5	0.7
108g	2	2	2	4	4

	(21-1)	(24-3)
Water	5.4	8.4
5x	8	8
25MM HP/G	12	12
25MM cap	9.6	8.4
SWA/Not I	0.5	0.7
100MM GTP	0.5	0.7
108g	4	4

5/13/95

TRANSFECTIONS

1. N-Rep + 21-1 + 24-3
 2. GP-Rep + 21-1 + 24-3
 3. HA-Rep + 21-1 + 24-3
 4. N
 5. GP
 6. HA
 7. 21-1 + 24-3
- } no 10 pul each
conch. see
gels
- GP-Rep (not used)

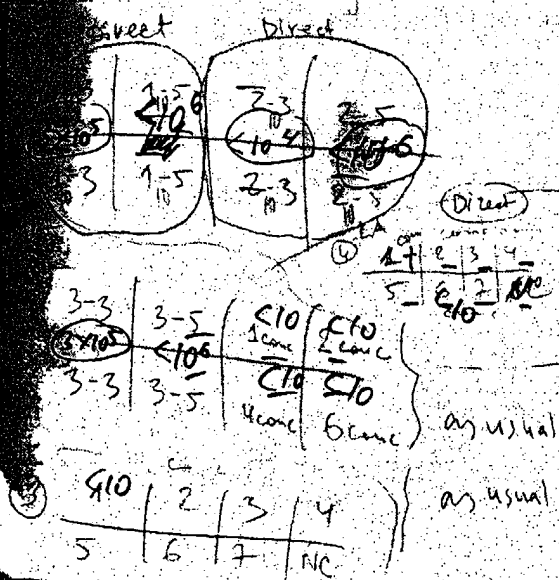


5/14/95

1
2
3
4
5
6
7

exposure
error

22 hr	p.e.	CSN	VE	HA	CPE 24 hr p.
		90%	70%	-	+/-
	pale	80-90% (??)	40%	-	+/-
	opaque	0-5%	70%	+	-
		50%	0	-	+/-
	pale	50% (?)	0	-	+/-
		0 !!!	0	+	-
		0 !!!	0	-	+/-



Western

a-L

-a-VEE (KMAF)

Block: 100 ml PBS:

 10% 10 g SKIM MILK
 0.5% 0.5 ml Tween 20

Wash: 2 x PBS

 0.5% 10g SKIM M
 0.025% 0.5 ml Tween 20

June 13, 1995

- ① Oligo synthesis for PCR mutagenesis
- THFECapsid 1) H141 → G141 (cap H141)
- 2) with Att II site
- universal capsid primer with Tth IIII site (capsid universal)

- 384 Sequences Listing -

(Version 1.02)

Name: CAPUT111

to Proline 141

1:24:30P, 8/13/95

Total bases: 84

 A- 8, G- 18, C- 18, T- 12, S- 8, S- 8, 7- 8, S- 8
 (Total bases: 84)

Seq: 1000000

Att II

OK

5' CCG TTA ACC GCG GCG AGA AGC TCG TTS TCG ATC TTS CCG

TCG ACA GCG ATC GCG 18'

- 384 Sequences Listing -

(Version 1.02)

Name: CAPUT111

1:28:10P, 8/13/95

Total bases: 84

 A- 18, G- 8, C- 7, T- 7, S- 8, S- 8, 7- 8, S- 8
 (Total bases: 84)

Seq: 1000000 Tth IIII

5' AAA CAG ATA CTA TCG TCG GCG AGA ATG TCG

2

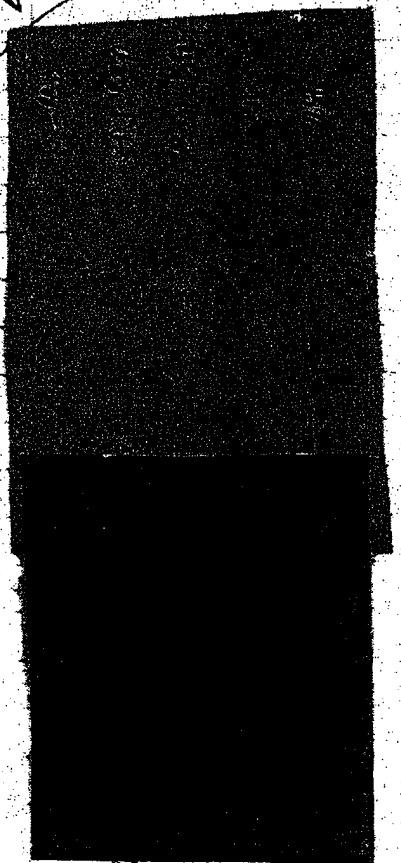
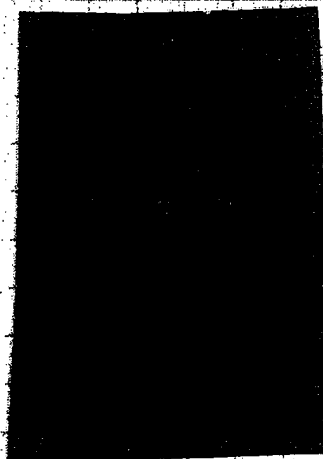
② In vitro transcription

N-lep, { were made (p. 125, in pencil)
 GPC-lep { should be mixed with
 HA-lep { other preparation made as in (p. 125, in pen)
 mix and use for transcription

21-1 { unsuccessful,
 24-3 { try 2nd of DNA

③ E. coli DNA transcription with

- 1) 21-1
- 2) 24-3



GTP cap
1:1.5

Mix

GTP cap
1:3

June 14, 1995

① in vitro transcription

water
 5x
 25 mM ATP-G
 15 mM cap

21-1

3.9

8

12

9.6

24-3

4.9

8

12

8.4

Note 2

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